Universidade do Minho
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Responses of aquatic decomposers to resource availability and increased temperature

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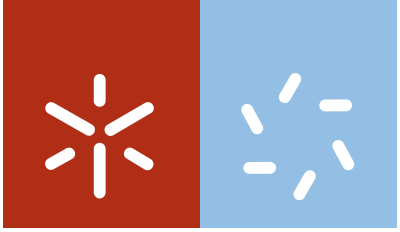

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Responses of aquatic decomposers to resource availability and increased temperature

PhD Thesis in Sciences
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Work Supervised By
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(Isabel Rodrigues Fernandes)

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Abstract

Freshwater ecosystems are very important to humans as running waters provide essential ecosystem goods and services to human well-being. Decomposition of allochthonous plant litter is a key process for the functioning of low-order forested streams, as plant litter constitutes the main source of food and energy for aquatic biota in detritus food webs. Microorganisms, including fungi and bacteria, play an important role in this process by conditioning leaf material resulting in a softer, palatable and nutritious food source for invertebrate shredders. Currently, freshwater ecosystems are heavily impacted by multiple stressors that include the increase of global temperature, the increase of nutrient loading and the loss of biodiversity. These stressors may occur simultaneously raising the need to further assess their interactive effects on key ecosystem processes, such as leaf decomposition in streams.

We examined the impacts of riparian plant diversity loss on diversity and activity of aquatic microbial decomposers. Mixtures of three leaf species (alder, oak and eucalyptus), commonly found in riparian corridors of Iberian streams, were immersed in a stream to allow microbial colonization. Simulation of species loss was done in microcosms by including a set of all leaf species, retrieved from the stream, and non-colonized leaves of three, two or one leaf species. Leaves were renewed every month throughout 6 months, and microbial inoculum was ensured by a set of colonized leaves from the previous month. To assess the potential impacts of time after leaf diversity loss on microbial diversity and activity, results were compared at 2 and 6 months after leaf diversity loss. We found that leaf diversity loss led to a decrease in molecular diversity of fungi and bacteria. Fungal biomass also decreased with leaf species loss for oak and eucalyptus leaves (lower quality; higher C:N ratio). Decomposition of alder and eucalyptus leaves was affected by leaf species identity, mainly after 6 months of diversity loss. Results suggest that effects of leaf diversity on microbial decomposers depended on leaf species number and also on which species were lost from the system, especially after longer times.

Besides affecting microbial activity, we expected that changes in litter diversity would affect higher trophic levels dependent on plant litter. To assess the potential impacts of time after leaf diversity loss on the feeding behaviour and elemental body composition of a stream invertebrate shredder, leaves subjected to leaf species loss simulation after 2 and 6 months were used to feed an invertebrate shredder. We found that the number and identity of leaf species affected leaf consumption and FPOM production by a common invertebrate shredder. Moreover, time after leaf diversity loss increased the positive diversity effects on leaf consumption and FPOM production. C and N composition of invertebrate body and of FPOM changed with the quality of leaf litter consumed by the shredder. Leaf consumption by the animals decreased linearly with the increase in C:N imbalance between leaf litter and invertebrate body. This suggests that riparian plant diversity can affect invertebrate shredder activity and the quality of resources (FPOM and invertebrate shredders) available to higher trophic levels.

Streams are exposed to multiple stressors making it difficult to predict the potential effects of changes in riparian vegetation on leaf decomposition and associated aquatic biota. Although eutrophication is a major problem in freshwater ecosystems, the knowledge of their potential interactive effects with leaf diversity on leaf decomposition is still scarce. To evaluate the potential interactive effects of stream eutrophication and leaf litter diversity we carried out a field experiment in which leaf mixtures up to 5 species were immersed in 6 streams along a gradient of eutrophication. Leaf species identity affected leaf mass loss, and fungal and invertebrate biomass on leaves. Leaf mass loss was higher in leaf mixtures than in single leaf species. N immobilization was higher in moderately and highly eutrophic streams and in leaves with the lowest initial N concentration. In general, a positive linear relationship between initial N concentration in leaves and leaf mass loss was found, and the slopes of the relationship increased with increasing eutrophication. Positive leaf diversity effects were observed because decomposition of leaf mixtures was higher than that expected from decomposition of individual leaf species. However, these effects were lost with increasing levels of eutrophication. This suggests that nutrient concentration in streams may modulate the effects of leaf diversity on leaf litter decomposition by enhancing the effects of leaf quality and attenuating the effects of leaf species number.

Global warming is an expected consequence of the ongoing climate change whose effects may interact with changes in riparian vegetation and eutrophication, further disturbing freshwater biota and ecosystem functioning. So, we evaluated the effects of litter quality (alder and oak, as high and low quality leaf types, respectively), increased inorganic nutrient concentration and increased stream water temperature on leaf decomposition and activity of the associated microbes. For that, alder and oak leaves naturally colonized in a stream were exposed in microcosms to a gradient of eutrophication under two temperatures: 12 ºC, a temperature typically found in Iberian streams in autumn and 18 ºC to simulate a warming scenario. Nitrogen immobilization in leaves increased with N concentration and temperature in the stream water, but was higher in alder than in oak leaves. In general, microbial activity increased asymptotically with N concentration in the stream water, following a Michaelis-Menten kinetic. Increased temperature led to an increase in maximum fungal activity. Interestingly, temperature potentiated the effects of nutrients because at increased temperature maximum fungal activity was achieved with lower N concentrations, especially on alder leaves (higher quality). This suggests that oligotrophic streams may be particularly vulnerable to increases in temperature because higher microbial activities might lead to faster organic matter turnover, especially in streams bordered by riparian vegetation delivering high quality leaf litter.

Resumo

Os ecossistemas de água doce são muito importantes pois providenciam bens e serviços essenciais ao bem-estar humano. A decomposição de detritos vegetais alóctones é um processo chave para o funcionamento dos ribeiros florestados, constituindo os detritos a principal fonte de alimento para os organismos aquáticos. Os microrganismos, incluindo fungos e bactérias, desempenham um papel importante neste processo, condicionando as folhas que se tornam uma fonte de alimento mais nutritiva para os invertebrados detritívoros. Atualmente, os ecossistemas de água doce estão muito alterados devido à ação de agentes múltiplos de stress, incluindo o aumento da temperatura, o aumento da carga de nutrientes nos rios e a perda de biodiversidade. Estes stressores podem ocorrer simultaneamente pelo que se torna importante avaliar os seus efeitos interativos em processos chave do ecossistema, como é o caso da decomposição de detritos em rios.

Primeiramente, foi avaliado o impacto da perda de diversidade da vegetação ribeirinha na diversidade e atividade de decompositores microbianos aquáticos. Misturas de três espécies de folhas (amieiro, carvalho e eucalipto), vulgarmente encontradas nos corredores ribeirinhos Ibéricos, foram imersas num rio para permitir a colonização microbiana. A perda de espécies foi simulada em microcosmos juntando conjuntos das 3 espécies de folhas, retirados do rio, a conjuntos com 3, 2 ou 1 espécie de folhas não colonizadas. As folhas foram renovadas todos os meses durante 6 meses, e o inóculo microbiano foi assegurado usando um conjunto de folhas colonizadas no mês anterior. Para avaliar os impactos do tempo após a perda de diversidade de folhas na diversidade e atividade microbianas, os resultados foram comparados aos 2 e 6 meses após a simulação da perda de espécies. A perda de espécies de folhas levou a uma diminuição da diversidade de fungos e bactérias. A biomassa de fungos diminuiu com a perda de espécies de folhas, especialmente nas folhas de carvalho e eucalipto (menor qualidade; maior razão C:N). A decomposição do amieiro e do eucalipto foi afetada pela identidade das espécies de folhas, especialmente após 6 meses da perda de espécies. Os resultados sugerem que os efeitos da diversidade de folhada nos decompositores microbianos dependeram do número de espécies e da identidade da espécie que foi perdida, especialmente a tempos mais longos.

Para além de afetar a atividade microbiana, espera-se que a alteração da diversidade da folhada possa afetar níveis tróficos superiores. Para avaliar os efeitos do tempo após a perda de diversidade na alimentação e na composição elementar do corpo de um invertebrado detritívoro, os animais foram alimentados com as folhas sujeitas aos tratamentos de simulação de perda de espécies de folhas após 2 e 6 meses. O número e a identidade das espécies de folhas afetaram o consumo de folha e a produção de matéria orgânica particulada fina (FPOM). O tempo após a perda da diversidade de folhas aumentou os efeitos positivos da diversidade no consumo de folha e na produção de FPOM. A composição do corpo do invertebrado e da FPOM em termos de C e N alterou-se com a qualidade da folha consumida. O consumo de folha diminuiu linearmente com o aumento do desequilíbrio na razão C:N entre a folhada e o corpo do animal. Isto sugere que a

diversidade de plantas ribeirinhas pode afetar a atividade dos invertebrados detritívoros e a qualidade dos recursos (FPOM e invertebrados detritívoros) para níveis tróficos superiores.

O facto de os rios estarem expostos a muitos agentes de stress pode dificultar a previsão dos efeitos das alterações da vegetação ribeirinha no processo de decomposição da folhada. Apesar da eutrofização ser um problema nos ecossistemas de água doce, o conhecimento sobre os seus efeitos interativos com a diversidade da vegetação ribeirinha são ainda escassos. Para colmatar essa lacuna, foi feita uma experiência de campo na qual misturas até 5 espécies de folhas foram imersas em 6 ribeiros ao longo de um gradiente de eutrofização. A identidade das espécies de folha afetaram a decomposição da folhada, e a biomassa de fungos e de invertebrados. A decomposição da folhada foi maior nas misturas de folhas do que nas espécies isoladas. A imobilização de N foi maior nos rios moderada e altamente eutrofizados e nas folhas com menor concentração inicial de N. No geral, foi observada uma relação linear positiva entre a concentração inicial de N nas folhas e a perda de massa foliar, e o declive da regressão aumentou com o aumento da eutrofização. Foram observados efeitos positivos da diversidade da folhada porque a decomposição nas misturas foi maior do que a esperada baseada na decomposição das espécies isoladamente. Contudo, estes efeitos positivos foram perdidos com o aumento da eutrofização. Isto sugere que a concentração de nutrientes nos rios pode modelar os efeitos da diversidade da folhada na decomposição das folhas aumentando os efeitos da qualidade das folhas e diminuindo os efeitos do número de espécies.

O aquecimento global é uma consequência esperada das atuais alterações climáticas globais que pode interagir com as alterações da vegetação ribeirinha e com a eutrofização, perturbando ainda mais os ecossistemas de água doce. Por isso, foram avaliados os efeitos da qualidade da folhada (amieiro e carvalho, folha de maior e menor qualidade, respetivamente), do aumento da concentração de nutrientes e da temperatura da água na decomposição de folhada e na atividade dos microrganismos. Folhas de amieiro e de carvalho, naturalmente colonizadas num rio, foram transferidas para microcosmos e expostas a um gradiente de eutrofização sob 2 temperaturas: 12 ºC, uma temperatura típica de ribeiros Ibéricos no outono e 18 ºC para simular um cenário de aquecimento global. A imobilização de N nas folhas aumentou com o aumento da concentração de N e da temperatura na água, e foi maior no amieiro do que no carvalho. No geral, a atividade microbiana aumentou assimptoticamente com a concentração de N na água, seguindo uma cinética de Michaelis-Menten. A temperatura mais elevada levou a um aumento da atividade máxima dos fungos. Curiosamente, a temperatura potenciou os efeitos dos nutrientes porque à temperatura mais alta a atividade fúngica máxima foi atingida com uma menor concentração de N na água, especialmente nas folhas de amieiro (maior qualidade). Isto sugere que os ribeiros oligotróficos podem ser particularmente vulneráveis ao aumento da temperatura porque as maiores atividades microbianas podem levar a um rápido consumo da matéria orgânica, especialmente em ribeiros cuja vegetação ribeirinha produz folhada de maior qualidade.

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Chapter 1

General introduction

1.1. Anthropogenic impacts to freshwater biodiversity

Over the last decades, the increasing human impacts on our planet has led to a massive extinction of species (Chapin III *et al.*, 2000), being freshwater ecosystems one of the most endangered (Dudgeon *et al.*, 2006). Freshwaters support almost 6% of all described species (Dudgeon *et al.*, 2006). This is a disproportional large fraction of the world's total biodiversity taken into account that freshwaters take up only about 0.01% of the world's water (Gleick, 1996). For instance, freshwater macrophytes represent ca. 1% of the total number of vascular plants known to date and 9.5% of the total number of animal species recognised globally are supported by freshwaters (Balian *et al.*, 2008). Few data is available regarding other group of organisms (e.g. bacteria, viruses, protozoa, fungi, and algae), which biodiversity is still largely unknown (Balian *et al.*, 2008). This lack of knowledge about microbial diversity might be critical since microorganisms play a key role in several environmental services like nutrient recycling, water purification and carbon sequestration (Ducklow, 2008), and the high rates of species loss that we are facing nowadays might compromise the maintenance of important ecosystem services.

The main anthropogenic impacts threatening freshwater biodiversity includes overexploitation, water pollution, flow modification, destruction or degradation of habitat, and invasion by exotic species (Millennium Ecosystem Assessment, 2005; Dudgeon *et al.*, 2006). A compilation of indicators on the progresses towards a reduction in the rate of biodiversity loss until 2010 showed that indicators of the state of biodiversity (e.g. extinction rates and population trends) are decreasing, while indicators of pressure on biodiversity (pollution, overexploitation, invasive species) are increasing (Butchart *et al.*, 2010). Climate change is also emerging in the last years as a major prompter of biodiversity alterations/loss (Bellard *et al.*, 2012). Climate change may lead to shifts in species physiology, phenology or range of distribution, or in the worst-case scenarios to species extinction (Bellard *et al.*, 2012). Scenarios point to a continuing decline of biodiversity during the 21st century, but there are also large uncertainties in projections and opportunities to implement better policies (Pereira *et al.*, 2010) to revert this trend.

The increasing human impacts on our planet has been leading to the extinction of a high number of species (Chapin III *et al.*, 2000), which has prompting research relating biodiversity and ecosystem processes over the last decades (for a review see Hooper *et al.*, 2005). A positive relationship between biodiversity and ecosystem functioning is the most common trend with meta-analyses comprising different trophic groups (producers, herbivores, detritivores and predators) from aquatic and

terrestrial ecosystems supporting these findings (Balvanera *et al.*, 2006; Cardinale *et al.*, 2006).

Several hypotheses have been proposed to describe the relationship between biodiversity and ecosystem functioning (reviewed by Johnson *et al.*, 1996). The stability-productivity hypothesis, proposed by MacArthur (1955), predicted a positive, linear relationship between species richness (trophically interacting species) and both productivity and the ability of ecosystems to recover after disturbance. Nevertheless, a linear correlation has rarely been observed (Tilman *et al.*, 1996; Hooper & Vitousek, 1997). The rivet hypothesis (Ehrlich & Ehrlich, 1981) predicts a positive non-linear relationship between species diversity and ecosystem functioning, likening species in an ecosystem to rivets holding an airplane together. The loss of some rivets is always negative, but beyond a critical threshold it may cause the airplane, or the ecosystem, to suddenly collapse. The redundancy hypothesis (Walker, 1992) predicts a positive and asymptotic relationship between species diversity and ecosystem function. Loss of species is not reflected in ecosystem function alteration as long as functional groups are well represented because ecological functions of lost species can be compensated by other species. Nevertheless, species that have a redundant role in an ecological process may have different traits to respond to environmental change (Fernandes *et al.*, 2011). So, maintaining high levels of species richness may provide long-term insurance to maintain ecosystem functions when environmental changes and fluctuations occur– insurance hypothesis (Yachi & Loreau, 1999). Sometimes, no relationship or an idiosyncratic relationship is observed between species richness and ecosystem functioning - idiosyncratic hypothesis (Lawton, 1994). This is because the effects of species diversity are dependent on the environmental context, on the species traits and the order by which species are lost from the system (Vitousek & Hooper, 1993; Lawton, 1994; Cardinale *et al.*, 2000). A large amount of studies on the relationships between species diversity and ecosystem functioning have been performed but because these relationships can be modulated by many factors (e.g. environmental context, Cardinale *et al.*, 2000; species traits, Spooner *et al.*, 2012) make them changeable and difficult to predict (Johnson *et al.*, 1996; Cardinale *et al.*, 2000).

1.2. Plant-litter decomposition in streams and associated aquatic biota

In low-order forested streams, the canopy of riparian vegetation reduces the amount of light that reaches the stream, reducing primary production (Benfield, 1996). Consequently, the allochthonous input of coarse particulate organic matter (CPOM)

from surrounding riparian vegetation becomes the main energy and nutrient source for detritus food webs (Webster & Benfield, 1986; Benfield, 1996; Suberkropp, 1998b). CPOM is mainly constituted by leaves that enter the streams (Abelho & Graça, 1998; Oelbermann & Gordon, 2000) and can be used by consumers and decomposers, stored or transported downstream.

After immersion in the stream, leaves start to lose soluble organic and inorganic compounds in a process known as leaching (Webster & Benfield, 1986; Benfield, 1996). The rapid loss of leaf compounds, including soluble sugars and polyphenols (Suberkropp *et al.*, 1976), facilitates microbial conditioning, mainly by aquatic fungi and bacteria (Suberkropp, 1998b; Bärlocher, 2005; Gessner *et al.*, 2007). During conditioning, microorganisms improve leaf palatability and transform plant material into a more suitable and nutritious food source for invertebrate shredders (Suberkropp, 1998b; Graça, 2001). Leaf material is further fragmented due to invertebrate shredders feeding activity (Webster & Benfield, 1986; Graça & Canhoto, 2006). Also, physical fragmentation of leaf material can occur due to flowing water abrasion (Webster & Benfield, 1986; Graça & Canhoto, 2006). Although leaf decomposition events tend to occur sequentially in time, these stages can occur simultaneously; for instance, the mechanical and enzymatic activity of microorganisms and invertebrates on leaves may affect patterns of solute release (Gessner *et al.*, 1999). During decomposition, microorganisms and invertebrate shredders convert leaf litter into biomass, fine particulate organic matter (FPOM), dissolved organic matter (DOM) and CO_2 (Webster & Benfield, 1986; Gessner *et al.*, 1999).

When leaves fall into the streams they are usually colonized by terrestrial fungi and bacteria (Bärlocher & Kendrick, 1974; Suberkropp & Klug, 1976), but they are promptly replaced by aquatic fungi (Suberkropp, 1998b; Gessner *et al.*, 2007). The role of aquatic fungi in leaf litter decomposition in streams is great, constituting more than 90% of the total microbial biomass (Baldy *et al.*, 1995; Pascoal & Cássio, 2004).

Aquatic hyphomycetes are a phylogenetically heterogeneous group of fungi, being most species affiliated with Ascomycota (Shearer *et al.*, 2007). However, the majority of anamorph/teleomorph associations are still unknown, since the sexual state has been reported in only ca. 10% of the species (Bärlocher, 2007; Marvanová, 2007; Shearer *et al.*, 2007). These fungi are ubiquitous and have morphological and physiological adaptations to lotic environments that dictate their success as colonizers and decomposers of leaf litter (Suberkropp, 1998b; Bärlocher, 2005). Their high conidial production and germination rates, their conidial shapes

(tetraradiate or sigmoid) and their ability to produce mucilage at the end of conidial arms are among the morphological and physiological characteristics that allow an efficient dispersion and attachment of aquatic fungi to new substrata (Read *et al.*, 1992). Other physiological adaptations include their ability to produce a vast range of extracellular enzymes, with cellulolytic, pectinolytic and proteolytic activity, which attack plant cell-wall polysaccharides contributing to their decomposition (Suberkropp *et al.*, 1983; Chamier, 1985). Furthermore, aquatic hyphomycetes are able to remain active, grow and reproduce at relatively low temperatures typical of cold seasons in temperate climates (Suberkropp, 1984). Actually, they show great temperature plasticity as they have been found at temperatures ranging from 0 to 34 ºC (Rajashekhar & Kaveriappa, 2003; Nikolcheva & Bärlocher, 2005).

Other fungi like yeast and yeast-like organisms are commonly found in aquatic environments (Spencer *et al.*, 1970). Several species have been found associated with decomposing plant litter in streams (Sampaio *et al.*, 2001, 2004; Sampaio *et al.*, 2007). However, their role in leaf decomposition is probably minor since only few species are able to degrade cellulose (Dennis, 1972) and xylan (Biely *et al.*, 1978). The density of yeast and yeast-like fungi increase in later stages of litter decomposition (Sampaio *et al.*, 2001; Gonçalves *et al.*, 2006) and presumably they are opportunistic organisms that assimilate the substances released during the degradation of plant material by other fungi or bacteria (Sampaio *et al.*, 2001).

Bacteria are also part of the microbial communities associated with decomposing plant litter in streams (Suberkropp & Klug, 1976; Baldy *et al.*, 1995; Duarte *et al.*, 2010). Based on data from microbial biomass and productivity, bacteria appear to play a less important role than fungi during early stages of leaf decomposition (Weyers & Suberkropp, 1996; Pascoal & Cássio, 2004). Indeed, bacterial biomass tends to predominate on FPOM, while fungal biomass predominates in CPOM like leaves and wood debris (Findlay *et al.*, 2002). Invertebrates can derive a large amount of carbon from bacteria, and so bacteria may also represent an important source of energy and nutrients to higher trophic levels in detritus-based food webs (Hall & Meyer, 1998).

Fungi and bacteria on decomposing leaves are reported to interact both positively (Wohl & McArthur, 2001; Romaní *et al.*, 2006) and negatively (Wohl & McArthur, 2001; Gulis & Suberkropp, 2003b; Mille-Lindblom & Tranvik, 2003; Romaní *et al.*, 2006); lack of significant interactions were also reported (Das *et al.*, 2012). Fungi and bacteria might compete for resources (Gulis & Suberkropp, 2003a; Mille-Lindblom *et al.*, 2006) and produce compounds capable of inhibiting each other (e.g. antibiotics produced by fungi; Gulis & Stephanovich, 1999). Bacteria might depend

more on fungal enzymatic activity, because bacterial enzymatic activities involved in the degradation of lignin and cellulose (phenol oxidase and cellobiohydrolase) appear to be very low (Romaní *et al.*, 2006).

Protozoa inhabit freshwaters and are very important grazers of microbes in aquatic environments (Finlay & Esteban, 1998). The role of protists in leaf decomposition in streams is almost unknown. However, higher leaf decomposition rates were observed in the presence of bacterivorous protists suggesting that they may play a role in plant-litter decomposition in streams (Ribblett *et al.*, 2005). It was hypothesized that grazing by protists could lead to a higher bacterial turnover rate, resulting in enhanced mineralization (Ribblett *et al.*, 2005). Risse-Buhl *et al*. (2012) found that protists positively affected leaf-associated community respiration, however protists had no effect on leaf mass loss.

Invertebrates can be classified according to their feeding behaviour into shredders, grazers or scrapers, collectors (both gatherers and filterers) and predators (Cummins, 1973; Tachet *et al.*, 2010). Among invertebrates, shredders feed on CPOM leading to the production of FPOM, which in turns serves as a food source for other invertebrates like the collectors (Wallace & Webster, 1996). The massive decrease of invertebrate populations in streams (by applying insecticide) was shown to significantly reduce leaf decomposition and FPOM export, proving the importance of invertebrate shredders in organic matter decomposition in streams (Cuffney *et al.*, 1990; Wallace *et al.*, 1995). Nevertheless, the role of shredders can be minor in large rivers (Chauvet *et al.*, 1993), tropical streams (Mathuriau & Chauvet, 2002) and polluted streams (Pascoal *et al.*, 2005a), where microorganisms seem to increase their contribution to leaf decomposition.

Invertebrates prefer to feed on microbially-colonized leaves (Kostalos & Seymour, 1976; Arsuffi & Suberkropp, 1985; Duarte *et al.*, 2012). Fungi, in particular, may improve litter quality to invertebrates since they may feed directly on fungal biomass or take benefit of the enzymatic ability of fungi to degrade complex components of leaf litter (Bärlocher & Kendrick, 1973; Suberkropp *et al.*, 1983; Bärlocher, 1985). In addition, shredders also show preferences for certain fungal species (Suberkropp *et al.*, 1983; Butler & Suberkropp, 1986; Arsuffi & Suberkropp, 1989; Graça *et al.*, 1994), confirming the importance of fungi in linking leaf litter and higher trophic levels in streams.

1.3. Litter decomposition in streams: microcosm versus field experiments

Major criticisms to microcosms are that they are too simplified, they do not represent natural spatial and temporal variation due to their small size and short duration, and that microcosm apparatus may give rise to artefacts (Carpenter, 1996; Fraser, 1999; Drake & Kramer, 2012). The main advantages pointed to the use of microcosms comprise the control over environmental variables, ease of replication, and the ability to control the variables under study (Fraser & Keddy, 1997; Fraser, 1999). In spite of all the criticisms to the limited realism and scope of microcosms, they have been indicated as a valid tool to identify, explore and test for the mechanisms behind ecological processes (Benton *et al.*, 2007; Drake & Kramer, 2012).

Microcosms can help to understand the effects of certain environmental variables on leaf-litter decomposition in streams that are very difficult to study in the field. For instance, studies testing for the effects of temperature in the field are scarce due to the difficulties to find a range of temperatures in the same location (natural experiments, Friberg *et al.*, 2009) or to warm a portion of the stream (field manipulations, Bärlocher *et al.*, 2008; http://www1.ci.uc.pt/imar/ribeiras/index.php).

Actually, microcosms have proved useful for testing effects of temperature on leaf-litter decomposition and associated communities (Dang *et al.*, 2009; Fernandes *et al.*, 2009) or on the physiology of aquatic fungi (Chauvet & Suberkropp, 1998; Rajashekhar & Kaveriappa, 2000). Another example is the investigation on the effects of nutrient loads on leaf decomposition and/or associated communities in streams by comparing effects in streams with different nutrient concentrations (Pascoal *et al.*, 2003; Gulis *et al.*, 2006; Baldy *et al.*, 2007) or by adding nutrients to the stream (Grattan & Suberkropp, 2001; Gulis & Suberkropp, 2004; Abelho & Graça, 2006; Ferreira *et al.*, 2006; Ferreiro *et al.*, 2011). However, due to the high variability that can occur in the field, several studies on effect of nutrient were done in microcosms (Suberkropp, 1998a; Sridhar & Bärlocher, 2000; Gulis & Suberkropp, 2003b). Also, when testing for interactive effects that are difficult to reproduce in the field, microcosms can become a suitable option (e.g. nutrient and species diversity, Bärlocher & Corkum, 2003; nutrient and temperature, Ferreira & Chauvet, 2011b; leaf diversity and temperature, Fernandes *et al.*, 2012). Thus, microcosms can be a useful tool to investigate global ecological problems (Benton *et al.*, 2007), such as ecosystem responses to climate change, consequences of alterations in biodiversity to ecosystem processes or increased nutrient loads, complementing field studies.

1.4. Global warming and litter decomposition in streams

Global warming emerges as a consequence of the ongoing climate change. Earth is predicted to face an average increase in air temperature that might vary according to the possible emission scenarios of greenhouse gases from +1.8 ºC (1.1 to 2.9 °C) to +4.0 °C (2.4 to 6.4 °C) until 2100 (Meehl *et al.*, 2007). Besides changes in average temperature, diurnal range of temperatures might change and the predictions point to more warm days and fewer cold nights (Rodríguez-Puebla *et al.*, 2010). Increase in air temperature might result in increased water temperature, which is already occurring (Kaushal *et al.*, 2010). Organisms are vulnerable to temperature increases and their responses may encompass changes in physiology, phenology, distribution and adaptation (Hughes, 2000). A meta-analysis assessing the effect of global warming on stream organisms showed that fish communities of large rivers in France undergoing various anthropogenic pressures presented increased proportions of warm-water species during the last 15-25 years (Daufresne & Boët, 2007). Almost 60% of European stream macroinvertebrates belonging to 12 orders may face a decrease in the amount of climatically suitable areas, which are expected to shift north and east (Domisch *et al.*, 2013). Increased temperatures may consequently result in altered community structure and activity with potential consequences to ecosystem functioning.

Temperature is expected to alter interspecific relationships among aquatic fungi. Indeed, within a species' optimal temperature range, the growth of that species increased with higher fungal diversity, while outside this range growth decreased with diversity (Duarte *et al.*, 2013). This suggests that fungi adapted to a certain temperature range might not cope with increased temperature leading to shifts in species dominance within communities. Actually, increased temperature changed fungal community composition (Dang *et al.*, 2009; Fernandes *et al.*, 2009; Fernandes *et al.*, 2012) and decreased fungal species diversity associated with leaf-litter decomposing in streams (Bärlocher *et al.*, 2008; Fernandes *et al.*, 2012).

Generally, metabolic rates increase with increasing temperature until a certain level above which further increase in temperature will inhibit organism metabolism (Brown *et al.*, 2004). Increased water temperature in streams from 2.2-4.3 ºC has been predicted to increase bacterial respiration from 26 to 63% (Sand-Jensen *et al.*, 2007). An increase in temperature by 5-10 ºC stimulated microbially-driven litter decomposition in stream microcosms (Dang *et al.*, 2009; Fernandes *et al.*, 2009; Fernandes *et al.*, 2012). Also, in the hyporheic zone of a stream, whose temperature was artificially raised by 4.3 ºC, litter decomposition increased (Bärlocher *et al.*,

2008). Litter decomposition mediated by invertebrates might also be affected by temperature. For instance, consumption rates of the shredder larvae *Sericostoma vittatum* increased with temperature until 13.7 to 16.7 ºC depending on the diet (González & Graça, 2003). Also, in 10 first-order Icelandic streams, affected by geothermal activity, macroinvertebrate density and leaf litter decomposition in fine- and coarse-mesh bags increased significantly with the increase in temperature from 6 to 23 ºC (Friberg *et al.*, 2009). However, data from an experiment across a latitudinal temperature gradient suggested that climate warming will likely accelerate microbial litter decomposition in the same proportion as it will decline detritivore-mediated decomposition, resulting in overall unchanged decomposition rates (Boyero *et al.*, 2011). By measuring whole-system metabolism in naturally heated geothermal streams in Iceland (5-25 ºC), Demars *et al*. (2011) found that whole-stream nutrient uptake was significantly faster in the warm streams than in the cold streams and the global stream carbon emission to the atmosphere would nearly double with a 5 ºC warming. Nevertheless, warming might interact with other environmental factors making it difficult to predict the overall effects of global warming on ecosystem functioning.

1.5. Eutrophication and litter decomposition

A recent synthesis on global threats to human water security and river biodiversity showed that nearly 80% of the world's population is subjected to high levels of threat (Vörösmarty *et al.*, 2010). In that study, water pollution, particularly nutrient loading (nitrogen and phosphorus), emerged as the second most important threat to human water security and biodiversity (Vörösmarty *et al.*, 2010). This is of great concern because drivers of eutrophication are expected to rise due to growth of world population, which will put more pressure on the productive capacity of agriculture and industry, and will enhance consumption of fossil fuel energy leading to increased release of nitrogen oxides (NO_x) to the environment (Selman & Greenhalgh, 2009). In addition, climate change predicts changes in precipitation regimes that may affect nutrient concentration in streams. Higher precipitation can raise surface runoff and erosion leading to increased nutrient concentrations in water bodies (Jeppesen *et al.*, 2009; Jeppesen *et al.*, 2011). Furthermore, decreased summer flow and increased evapotranspiration may result in less dilution of nutrient inputs and higher nutrient concentrations in water bodies (Murdoch *et al.*, 2000; Whitehead *et al.*, 2009). In both cases, streams might be facing higher levels of nutrients in the future.

Fungi associated with decomposing leaves in streams may obtain part of the nutrients they need (e.g., nitrogen and phosphorus) from the water, suggesting that stream water chemistry can be an important regulator of microbially-driven leaf decomposition (Suberkropp & Chauvet, 1995). Plant-litter decomposition may be stimulated by nutrient enrichment in streams (Sridhar & Bärlocher, 2000; Gulis & Suberkropp, 2003c; Pascoal *et al.*, 2005a; Gulis *et al.*, 2006), which can result from increased microbial activity (Sridhar & Bärlocher, 2000; Gulis & Suberkropp, 2003a, 2003c; Duarte *et al.*, 2009). Invertebrate density and biomass, as well as fungal diversity and sporulation may also respond positively to stream nutrient enrichment (Gulis & Suberkropp, 2004; Greenwood *et al.*, 2007; Chung & Suberkropp, 2008). Contrarily, invertebrate diversity may decrease as sensitive taxa of invertebrate shredders decline under eutrophic conditions (Pascoal *et al.*, 2005a). Still, some studies have found no effect of nutrient enrichment on leaf decomposition by microbes or microbes and invertebrates (Royer & Minshall, 2001; Abelho & Graça, 2006; Ferreiro *et al.*, 2011). A reduction in fungal diversity and reproduction may occur in streams with high nutrient concentration without any change in leaf decomposition rate probably as a result of functional redundancy among aquatic fungi (Raviraja *et al.*, 1998; Pascoal *et al.*, 2005b; Sridhar *et al.*, 2009). Nevertheless, in highly eutrophic streams the low dissolved oxygen concentration in water and sedimentation (Pascoal *et al.*, 2005a), or high concentrations of ammonia and nitrites (Lecerf *et al.*, 2006; Duarte *et al.*, 2009) may have deleterious effects on fungal and/or invertebrate activity and, consequently, reduce litter decomposition (Pascoal *et al.*, 2005a; Lecerf *et al.*, 2006; Duarte *et al.*, 2009).

Microbial nutrient demands can be fulfilled at relatively low levels and further increases in the nutrient availability may not result in enhanced fungal activity (Ferreira *et al.*, 2006; Artigas *et al.*, 2008). Indeed, nutrient concentration in the stream water showed an asymptotic relationship with leaf decomposition rates and associated microbial activity (e.g., fungal sporulation and biomass) or invertebrate biomass (Rosemond *et al.*, 2002; Ferreira *et al.*, 2006; Gulis *et al.*, 2006). Nevertheless, nutrient concentrations above certain levels can become toxic to biota and inhibit biological processes. This was recently confirmed in a experiment across 100 European streams spanning a large nutrient range in which a hump-shaped curve described the relationship between nutrients in the stream water and leaf-litter decomposition (Woodward *et al.*, 2012).

1.6. Riparian plant diversity and litter decomposition in streams

Current estimates of biodiversity for the future are very variable, depending on the method and metrics used to assess biodiversity loss, the taxonomic group under analysis and, also, on the considered spatial and temporal scales (Bellard *et al.*, 2012). However, the majority of models indicate alarming consequences for future biodiversity, with the worst-case scenario leading to extinction rates that would be qualified as the sixth mass extinction in the earth's history (Bellard *et al.*, 2012). Projected climate warming will potentially have profound effects on the earth's biota, being expected large redistribution of tree plant species (Iverson & Prasad, 1998; Bakkenes *et al.*, 2002). Moreover, agricultural and industrial activities, increased urban settlements, the introduction of exotic species (e.g. eucalypt in Iberian Peninsula) or the spread of invasive species (e.g. Japanese knotweed in Europe) are decreasing forest tree diversity throughout the world (Graça *et al.*, 2002; Foley *et al.*, 2005; Lecerf *et al.*, 2007a; Haines-Young, 2009). For instance, in Portugal, eucalyptus plantations constitutes 26% of the forest cover and its total area increased 13% between 1995 and 2010 (ICNF, 2013). Changes in riparian vegetation may change the amount and quality of litter inputs to the streams (Webster *et al.*, 1990), potentially affecting aquatic biota dependent on this source of food and energy and ultimately affecting ecosystem functioning.

One could expect that a more diverse plant litter in streams would provide more niches to aquatic biota by supplying different food and habitat. Actually, the composition and diversity of riparian plant species affect the diversity of aquatic fungi (Laitung & Chauvet, 2005; Lecerf *et al.*, 2005). Also, the abundance of invertebrate taxa was lower in streams running through eucalypt plantations comparatively to mixed deciduous forests (Abelho & Graça, 1996). Nevertheless, this is not always the case because similar communities of invertebrates and microbes were found in streams running through forests with different riparian composition (coniferous, deciduous and mixed) (Kominoski *et al.*, 2011).

Besides affecting aquatic communities, changes in litter diversity may also affect plant litter decomposition. Litter diversity effects on litter decomposition can be assessed by comparing litter decomposition in the mixtures with that expected from the sum of single litter species (Hui & Jackson, 2009). When litter decomposition in mixtures is higher than that expected based on single species decomposition there is evidence of synergistic effects, while the opposite is indicative of antagonistic effects of litter diversity (Hui & Jackson, 2009). Overall, studies in freshwaters have pointed to the predominance of synergistic effects of litter diversity on decomposition

(Lecerf *et al.*, 2011). Moreover, the composition of litter mixtures appears to have a greater role in leaf decomposition in streams than the number of litter species (Swan & Palmer, 2004, 2006a; Lecerf *et al.*, 2007b; Ferreira *et al.*, 2012) with the effects of litter identity being explained by differences in litter quality (Lecerf & Chauvet, 2008; Schindler & Gessner, 2009; Fernandes *et al.*, 2012; Ferreira *et al.*, 2012). Leaves with high concentration of nutrients (e.g., nitrogen, phosphorus) and lower content of structural compounds (e.g., lignin, cellulose) and secondary compounds (e.g., waxes, cutins, tannins, essential oils) are considered of high quality for fungi and invertebrates, resulting in faster leaf decomposition (Webster & Benfield, 1986; Canhoto & Graça, 1996; Canhoto & Graça, 1999; Fernandes *et al.*, 2012). The preferential feeding on specific leaf types may result in altered leaf decomposition in leaf mixtures compared with that expected based on single leaf species (Swan & Palmer, 2006b). Also, leaves might be used to other purposes like construction of cases (Davies & Boulton, 2009; Sanpera-Calbet *et al.*, 2009), or as a refuge. As a result, we might expect litter diversity effects to be stronger in litter mixtures made of functionally dissimilar species (Lecerf *et al.*, 2011).

Because riparian tree species can deliver leaf litter with different chemical composition in streams, alterations in riparian plant diversity or composition may change the quality of available resources to aquatic biota. According to the ecological stoichiometry theory, consumers tend to maintain their elemental body composition constant (Sterner & Elser, 2002). Consumers usually have lower C:nutrient ratios than their food resources (Cross *et al.*, 2005; Hladyz *et al.*, 2009), resulting in elemental imbalances. Changes in litter quality may result in alteration of elemental imbalances between food resources and consumer requirements, potentially affecting the activity of aquatic biota and leaf decomposition in streams (Frost *et al.*, 2006; Hladyz *et al.*, 2009).

1.7. Global change and litter decomposition in streams

Ongoing global changes include the increase in global temperature, the increase in nutrient loading in freshwaters and the loss of biodiversity, which may occur simultaneously making it difficult to predict their combined effects on key ecosystem processes such as leaf decomposition in streams.

The direction and magnitude of litter diversity effects on plant-litter decomposition was found to change upon differences in temperature/seasonality from i) no effects of litter mixtures on decomposition in autumn to antagonistic effects in summer (Swan & Palmer, 2004); ii) positive litter diversity effects in a warmer stream in

France to null effects in a cooler stream in Romania (Lecerf *et al.*, 2007b); and iii) antagonistic or null effects at lowest and highest temperatures, compared to synergistic effects at intermediate temperatures (6-10 ºC) (Lecerf *et al.*, 2011). More recently, temperature was also found to modulate the effects of litter quality on microbially-mediated decomposition of plant litter in streams (Fernandes *et al.*, 2012). Nutrient concentration in streams alters litter diversity effects on leaf decomposition because the antagonistic effects observed in a reference stream were suppressed in a nutrient-enriched stream (Rosemond *et al.*, 2010). Moreover, Lecerf *et al.* (2011) observed an increase in the frequency of synergistic effects of litter mixture on litter decomposition with incubation time. They argued that the effects of riparian plant diversity on carbon and nutrient dynamics could be better inferred from long-duration experiments by manipulating functional diversity in ecosystems with different characteristics (Lecerf *et al.*, 2011).

Future increases in atmospheric CO_2 might lead to a decrease in litter quality because a decrease in nutrient concentration and an increase in structural and secondary compounds was observed in leaves grown under increased CO_2 concentration compared to leaves grown under ambient CO_2 concentration (Norby *et al.*, 2001; Stiling & Cornelissen, 2007). The interactive effects of increased temperature and altered litter quality (due to changes in atmospheric CO_2) suggested that the performance of fungi and invertebrates might be more controlled by water temperature than by minor changes in litter quality (Ferreira *et al.*, 2010; Ferreira & Chauvet, 2011a). However, increased temperature may interact synergistically with the increase of nutrient concentration in streams resulting in faster microbial activity and leaf decomposition (Ferreira & Chauvet, 2011b).

We could expect that organisms associated with low quality litter would benefit from nutrients in the stream water, leading to higher activities. Nevertheless, no consensus was reached on the interactive effects of litter quality and nutrient concentration in streams because effects of nutrient enrichment were more pronounced either on low quality leaf types (Gulis *et al.*, 2006) or on high quality leaf types (Ardón *et al.*, 2006; Ardón & Pringle, 2007). Overall, freshwaters are exposed to multiple stressors whose interactions might result in “ecological surprises” (Ormerod *et al.*, 2010) raising the need to further study their interactive effects.

1.8. Aim and outline of the thesis

In low-order forest streams, plant-litter decomposition is mainly driven by microorganisms and invertebrates. This key ecosystem process links riparian

vegetation to higher trophic levels and depends on several environmental factors. Freshwater ecosystems are increasingly threatened by human activities. Nutrient enrichment and alterations in riparian vegetation are among the major threats to streams. In this study, the interactive effects of riparian plant diversity, increasing nutrient loads and warming temperature were investigated on leaf-litter decomposition and the associated biota in streams to better understand ecosystem functioning under current global pressures.

Chapter 1 provides an overview on the relationship between biodiversity and ecosystem functioning, and on the major threats to freshwater biodiversity. Particular importance is given to leaf decomposition in freshwaters and associated communities. The major impacts of warming, eutrophication and plant diversity loss to leaf decomposition in streams and associated communities are addressed.

In the two subsequent chapters, we used a microcosm approach to assess the impacts of riparian plant diversity loss on microbial activity and diversity (Chapter 2), and on the feeding behaviour and body composition of a stream invertebrate shredder (Chapter 3). For that, leaves colonized in a mixed-forest stream were used to simulate leaf species loss over a period of 6 months and were further used to feed invertebrate shredders. In Chapter 4, a field experiment was conducted to test the interactive effects of leaf diversity and eutrophication on leaf decomposition and associated biota. Combinations of 1-5 leaf species commonly found in Iberian streams were immersed for 38 days in 6 streams along a gradient of eutrophication. Effects were assessed on leaf decomposition, on fungal and invertebrate biomass, on the structure of fungal and invertebrate communities, and on nitrogen immobilization on leaves. In Chapter 5, alder and oak leaves were colonized in a reference stream and used in microcosms to modulate the effects of nutrient concentration in water at two temperatures (12 and 18 ºC) under the hypothesis that temperature and litter quality would change the effects of nutrient enrichment on microbially-driven leaf decomposition. In Chapter 6, the main conclusions are presented to provide a global perspective of the work and possible lines for future research.

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Chapter 2

Effects of riparian plant diversity loss on aquatic microbial decomposers become more pronounced at longer times

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Effects of riparian plant diversity loss on aquatic microbial decomposers become more pronounced at longer times

Submitted

Abstract

We examined the potential long-term impacts of riparian plant diversity loss on diversity and activity of aquatic microbial decomposers. Microbial assemblages were obtained in a mixed-forest stream by immersion of mesh bags containing three leaf species (alder, oak and eucalyptus), commonly found in riparian corridors of Iberian streams. Simulation of species loss was done in microcosms by including a set of all leaf species, retrieved from the stream, and non-colonized leaves of three, two or one leaf species. Leaves were renewed every month throughout 6 months, and microbial inoculum was ensured by a set of colonized leaves from the previous month. Microbial diversity, leaf mass loss and fungal biomass were assessed at the 2nd and 6th month after plant species loss. Molecular diversity of fungi and bacteria, as the total number of operational taxonomic units per leaf diversity treatment, decreased with leaf diversity loss. Fungal biomass tended to decrease linearly with leaf species loss on oak and eucalyptus, suggesting more pronounced effects of leaf diversity on lower quality leaves. Decomposition of alder and eucalyptus leaves was affected by leaf species identity, mainly after long time of diversity loss. Leaf decomposition of alder decreased when mixed with eucalyptus, while decomposition of eucalyptus decreased in mixtures with oak. Results suggest that effects of leaf diversity on microbial decomposers depended on leaf species number and also on which species were lost from the system, especially after longer times. This may have implications for the management of riparian forests to maintain stream ecosystem functioning.

2.1. Introduction

Human activities are affecting freshwater ecosystems worldwide leading to irreversible changes in biotic communities and the processes they support (Vitousek *et al.*, 1997; Carpenter *et al.*, 2011). A key ecological process in freshwaters is plant-litter decomposition, which is driven by microorganisms and invertebrate detritivores (Graça & Canhoto, 2006; Gessner *et al.*, 2007). Both aquatic fungi and bacteria play a key role in organic-matter decomposition by converting plant litter into a more nutritious food source for invertebrate detritivores (Graça, 2001). Fungi have been recognized as the major microbial decomposers in streams accounting for more than 90% to the total biomass production on decomposing leaves (Pascoal & Cássio, 2004). However, the role of bacteria cannot be neglected because its contribution to plant-litter decomposition increases along time as smaller-size detritus are being produced (Findlay *et al.*, 2002; Pascoal *et al.*, 2005). Protozoa (e.g., ciliates) can exert a top-down predation pressure on aquatic bacterial communities (Dopheide *et al.*, 2011); however, its role in plant-litter decomposition remains practically unexplored (but see Ribblett *et al.*, 2005; Risse-Buhl *et al.*, 2012).

Due to the importance of plant-litter decomposition and its tractability in field and microcosm experiments, the scientific community is increasingly using this process to better understand the relationships between biodiversity and ecosystem functioning in freshwaters (Pascoal & Cássio, 2008; Gessner *et al.*, 2010). In fact, several studies have provided evidence of how leaf-litter decomposition is shaped by the diversity of resources (Lecerf *et al.*, 2005) and of consumers (fungi, Bärlocher & Corkum, 2003; Duarte *et al.*, 2006; Fernandes *et al.*, 2011; invertebrates, Jonsson & Malmqvist, 2000; Jonsson *et al.*, 2001; Reiss *et al.*, 2010).

Several studies indicate that composition and diversity of riparian plant species influence the diversity of aquatic fungi (Laitung & Chauvet, 2005; Lecerf *et al.*, 2005). The quality of plant-litter mixtures can also influence microbial biomass; for instance, the presence of high quality leaves of *Liriodendron tulipifera* in litter mixtures led to an increase of fungal and bacterial biomasses, while the presence of low quality leaves of *Rhododendron maximum* led to an opposite effect (Kominoski *et al.*, 2007). Most studies have focused on composite samples in litter mixtures (but see Kominoski *et al.*, 2009), but examining the effects of litter diversity loss within individual litter species might help to better understand the contribution of individual litter species for overall diversity effects on litter decomposition.

Microbes have faster growth rates than other organisms involved in plant-litter decomposition in streams, such as invertebrate detritivores. Generally, maximum fungal doubling times on leaf litter decomposing in streams range from 5 to 50 days (Gessner, 1997). Therefore, microbes can have several generations throughout plant-litter decomposition, and may show strong responses to alterations in litter diversity at relatively short-time scales.

The aim of this study was to examine how aquatic microbial decomposers respond to riparian plant diversity loss. We used a microcosm approach to monitor during 6 months the development of leaf-associated microbial assemblages after inducing the loss of plant species from the system. Microbial assemblages were obtained in a mixed-forest stream by immersion of a pool of three leaf species (alder, oak and eucalyptus) commonly found in the riparian corridors of Iberian streams. After 2 and 6 months, we assessed leaf mass loss, fungal biomass, and diversity of fungi, bacteria and ciliates associated with individual leaf species. We expected that plant-litter mixtures, containing litter species with different chemical composition, would provide better resources to support higher microbial diversity and/or activity. We also expected that any constrain to microbial biomass development or leaf decomposition of lower-quality leaf species could be overcome by the presence of high quality leaf species in mixtures.

2.2. Methods

2.2.1. Microbial colonization of leaves in a stream

In October 2009, leaves from alder (*Alnus glutinosa* (L.) Gaertn.), oak (*Quercus robur* L.) and eucalyptus (*Eucalyptus globulus* Labill.) were collected from trees immediately before abscission and dried at room temperature. Leaves were leached in deionised water, cut into disks, and 30 leaf disks of each of the three plant species were placed in fine-mesh bags (0.5-mm diameter pore size). On 28 October 2009, bags containing the leaf species mixtures were immersed in a mixed-forested stream in Portugal, the Estorãos stream (8.63800°W, 41.78194°N), to allow microbial colonization.

At the study site, the stream was about 0.5 m deep and 2.5 m wide, the stream bed was constituted by rocks, pebbles and gravel, and the riparian vegetation was dominated by *A. glutinosa*, *Q. robur* and *E. globulus*. During leaf colonization, stream water had on average (±SEM) a temperature of 14 ± 1.0 ºC, a pH of 5.9 ± 0.06, a conductivity of 31 µS cm^{-1} and a redox potential of 51 ± 1.5 mV (Multiline F/set 3 no. 400327; WTW, Weilheim, Germany). Nutrient concentrations in the

stream water were 0.30 ± 0.04 mg L^{-1} of $N\text{-}NO_3^-$, 0.003 ± 0.000 mg L^{-1} of $N\text{-}NO_2^-$, <0.01 mg L^{-1} of $N\text{-}NH_3$ and <0.003 $P\text{-}PO_4^{3-}$ (HACH kit, programs 351, 371, 385, and 490, respectively; HACH, Loveland, CO, USA).

2.2.2. Microcosm setup

After two weeks of stream immersion, leaf bags were brought to the laboratory, and 30 leaf disks from each of the three leaf species (a total of 90 leaf disks) were placed in microcosms (500 mL Erlenmeyer flask) containing 500 mL of sterile stream water. Simulation of leaf species loss was done in new microcosms containing non-colonized leaf disks of three, two or one leaf species enclosed in fine-mesh bags and 12 of the previously colonized leaf disks (4 of each plant species) to ensure microbial inoculum. To analyse long-term effects of leaf species loss, this procedure was repeated each 30th day during 6 months, and keeping leaf species proportion constant in each treatment. Four replicates were used per treatment. Microcosms were kept, aseptically, under aeration, with artificial light at 16 ºC, and stream water was renewed every 15 days. After 2 and 6 months, leaf disks were used to assess leaf mass loss, fungal biomass, and fungal, bacterial and ciliate diversity by denaturing gradient gel electrophoresis (DGGE), after PCR amplification of microbial DNA with specific primers targeting each microbial group.

2.2.3. Leaf mass loss

Leaf disks from all microcosms of each individual leaf species were freeze-dried for two days and weighed to the nearest 0.01 mg. Mass loss of each leaf species was expressed as percentage of the respective initial dry mass.

2.2.4. Fungal biomass

Fungal biomass was estimated from ergosterol concentration associated with decomposing leaf disks according to Gessner (2005). Briefly, lipids from each individual leaf species were extracted from sets of 6 leaf disks by heating (80 ºC for 30 minutes) in 8 g L^{-1} KOH-methanol. The lipid extract was purified by solid-phase extraction (Sep-Pak cartridges, Waters, Milford, MA) and ergosterol was quantified by high performance liquid chromatography (Beckmann Gold System, Brea, CA, USA), at 282 nm, using a LiChrospher RP18 column (250×4 mm, Merck). The system was run isocratically with methanol as mobile phase (1.4 mL min^{-1}, 33 ºC).

2.2.5. Microbial diversity

DNA was extracted from 4 freeze-dried disks of each leaf species with a soil DNA extraction kit (MoBio Laboratories, Solana Beach, California), according to the manufacturer instructions. The ITS2 region of fungal ribosomal DNA (rDNA) was amplified with the primer pair ITS3GC and ITS4 (Duarte *et al.*, 2010); V3 region of 16S bacterial rDNA was amplified with the primer pair 338GC and 518 (Duarte *et al.*, 2010); and 18S rDNA of ciliates was amplified with the primer pair 984GC and 1147 (adapted from Dopheide *et al.*, 2008). For PCR of fungal, bacterial and ciliate DNA, 2x of GoTaq® Green Master Mix (Promega), 0.4 µM of the appropriate primers and 1 to 10 µL of DNA (1-10 ng μL^{-1}) were used in a final volume of 25 µL.

PCRs were carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). The PCR program for bacteria and fungi was: initial denaturation at 95 ºC for 2 min; 36 cycles of denaturation at 95 ºC for 30 s; primer annealing at 55 ºC for 30 s and extension at 72 ºC for 1 min; and final extension at 72 ºC for 5 min (Duarte *et al.*, 2010). The PCR program for ciliates was: initial denaturation at 94 ºC for 5 min; 30 cycles of denaturation at 94 ºC for 45 s; primer annealing at 55 ºC for 60 s and extension at 72 ºC for 90 s; and final extension at 72 ºC for 7 min (Dopheide *et al.*, 2008).

DGGE analysis was performed using a DCodeTM Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). For fungi, 700 ng of the amplified DNA products with 380-400 bp were loaded on 8% (w/v) polyacrylamide gel in 1x Tris-acetate-EDTA (TAE 1x) with a denaturing gradient from 30 to 70% (100% denaturant corresponds to 40% formamide and 7 M urea). For bacteria, 700 ng of the amplified DNA products with ca. 200 bp were loaded on 8% (w/v) polyacrylamide gel in 1x TAE with a denaturing gradient from 40 to 75%. For ciliates, 700 ng of the amplified DNA products with 750-800 bp were loaded on 6% (w/v) polyacrilamide gel in 1x TAE with a denaturing gradient from 30 to 42.5%. Fungal and bacterial DNA was separated at 55 V and 56 ºC, while ciliate DNA was separated at 80 V and 60 ºC. All gels were run for 16 h. Gels were stained with 1x of GelStar (Lonza) for 10 min, and gel images captured under UV light in a gel documentation system (GenoSmart; VWR).

2.2.6. Nutrient content in leaves

To estimate initial carbon and nitrogen in leaves, samples of alder, oak and eucalyptus leaves were grounded with a ball mill and ca.100 mg of powdered leaf material was analyzed in a LECO-CNS 2000, using EDTA as a standard. Analyses

were done in CACTI – Centro de Apoio Científico e Tecnolóxico á Investigación – University of Vigo, Spain.

Initial quality of leaves as C:N ratio differed between the three leaf types as follows: alder (13.29 ± 0.26) < oak (19.69 ± 0.71) < eucalyptus (30.51 ± 0.26).

2.2.7. Statistical analyses

DGGE gels of microbial DNA were aligned and normalized using Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium), and each DGGE band was considered an operational taxonomic unit (OTU).

Linear regressions were used to establish the relationships between leaf species number and total number of OTUs of each microbial group per leaf treatment. The distribution of fungi, bacteria and ciliates associated with leaf species identity and number at each time (2 and 6 months) was analysed by Correspondence Analysis (CA, Legendre & Legendre, 1998) downweighting the contribution of rare species. Data of fungal, bacterial and ciliates communities' structure were based on OTUs, as the relative intensity of each band in DGGE fingerprinting. Data was square root transformed.

For each leaf type, a three-way nested ANOVA was used to test if leaf species number, leaf species identity (nested within species number) and time after diversity loss significantly affected leaf mass loss and fungal biomass (Zar, 2009). In punctual cases, in which diversity effects were marginally significant, a two-way nested ANOVA was done, testing for the effects of leaf species number and identity (nested with leaf species number) at each time separately. Because the experimental design was unbalanced, we applied Type III analyses of variance using the Variance Estimation and Precision (VEPAC) module in Statistica 8.0 (Statsoft, Tulsa, OK, USA). Differences between treatments were analysed using the Tukey-Krammer´s post-test, which is a modification of the Tukey´s post-test for unbalanced number of samples (Zar, 2009). Linear regressions were used to establish the relationships between leaf species number and fungal biomass for each leaf species.

Linear regressions were done in Prism 4.0 for Windows (GraphPad software Inc., San Diego, CA), analyses of variance were done in Statistica 8.0 for Windows (Statsoft, Inc., Tulsa, OK) and multivariate analyses were done in CANOCO 4.5 for Windows (Microcomputer Power, Ithaca, NY).

2.3. Results

2.3.1. Effects of plant litter diversity on microbial diversity

Molecular diversity of microbial communities on decomposing leaves showed a total of 41, 64 and 29 operational taxonomic units (OTUs) for fungi, bacteria and ciliates, respectively (Fig. 2.1). In a general way, communities of fungi, bacteria and ciliates on each leaf species differed between single- and mixed-species treatments (Fig. 2.1 and Fig. 2.2). The number of fungal OTUs on individual leaf species was higher in mixed-species treatments than in single species treatments, particularly at the longer time, i.e. after 6 months of leaf species loss (Fig. 2.2). Conversely, bacterial diversity on individual leaf species was generally higher in single-leaf species treatments than in mixed-species treatments (e.g. 46 OTUs on oak alone versus 35-38 OTUs on mixtures, after long time of leaf diversity loss). The diversity of ciliates appeared to decrease with time because lower number of OTUs was found after long time in microcosms (except for oak leaves). Similarly to that found for bacterial diversity, the number of ciliate OTUs on individual leaf species was higher in single leaf species treatments than in mixed species treatments (Fig. 2.2).

However, when taking into account the total number of OTUs associated with all leaf species composing a given mixture, positive relationships were found between leaf species number and fungal or bacterial diversity (linear regression, P=0.0003 and P=0.024, respectively; Fig. 2.3). For ciliates, that relationship was not significant (linear regression, P=0.065). The dependence of microbial diversity on leaf species number strongly increased from ciliates to bacteria to fungi (slopes were 2.0, 4.2 and 6.0 OTUs/unit of leaf species diversity, respectively).

CA ordination of fungal assemblages according to leaf species number, leaf species identity and time after leaf diversity loss showed that the 1st factor explained 20.8% of the total variance in fungal assemblages, and separated assemblages established at short time from those established at long time (Fig. 2.4a). The 2nd factor explained 15.2% of the total variance and distinguished assemblages according to leaf species identity and leaf species number, mainly separating fungal assemblages on 3 leaf species from the others. CA ordination of bacterial assemblages showed that the first two factors, explaining 24.5% of the total variance, separated assemblages on oak leaves from the others, and further discriminated assemblages according to leaf species number and the time after diversity loss (Fig. 2.4b). The 1st CA factor of ciliate assemblages explained 19.1% of the total variance and separated assemblages by the time after diversity loss,

while the 2nd factor explaining 15.1% of the total variance mainly distinguished assemblages according to leaf species number (Fig. 2.4c).

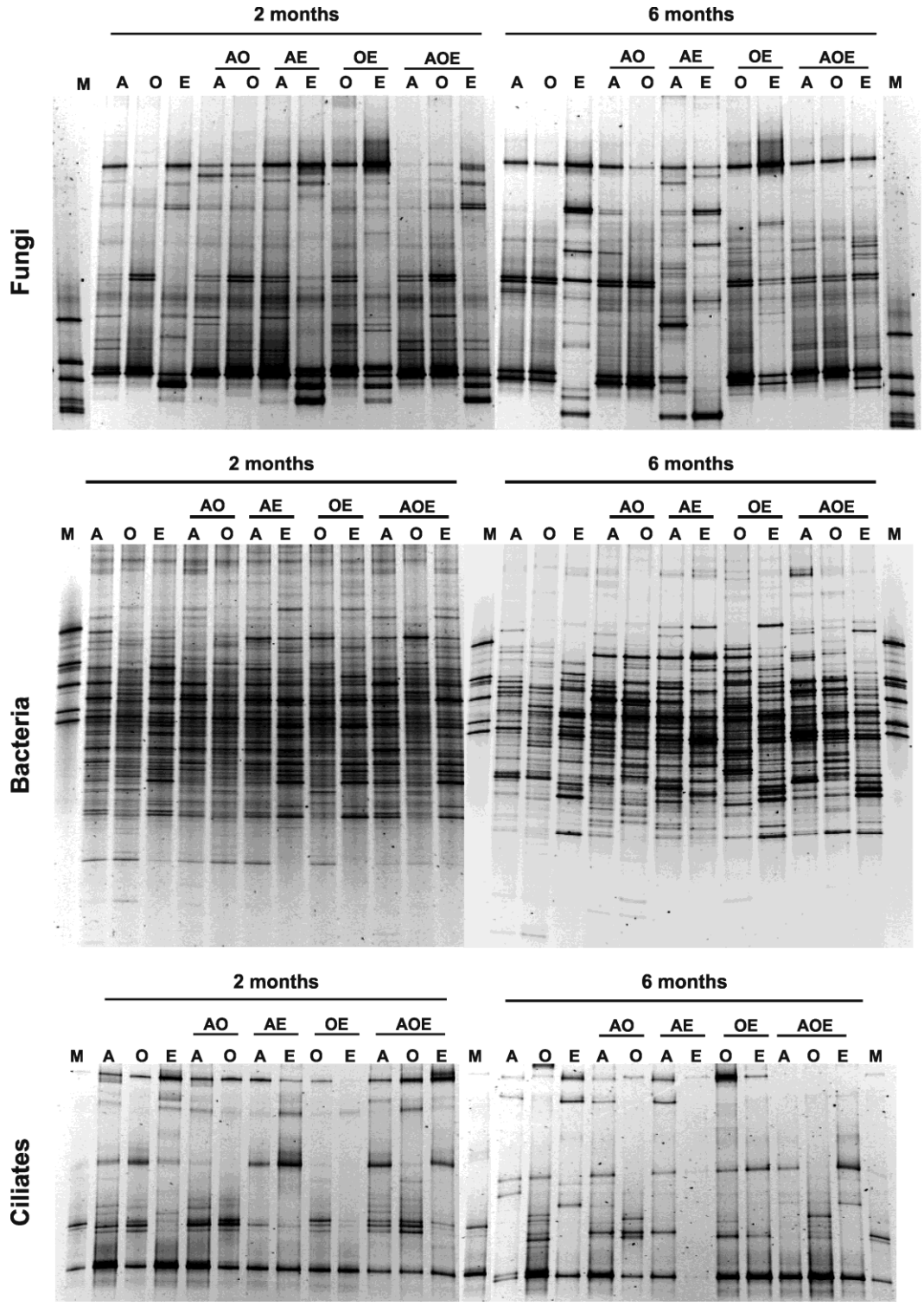

Figure 2.1 DGGE patterns of DNA of fungal, bacterial and ciliate assemblages on individual leaf species (A, *Alnus glutinosa*; O, *Quercus robur*; E, *Eucalyptus globulus*) from single- and mixed-leaf species treatments, after short (2 months) and long time (6 months) of leaf diversity loss. M, marker used to align different gels belonging to the same microbial group.

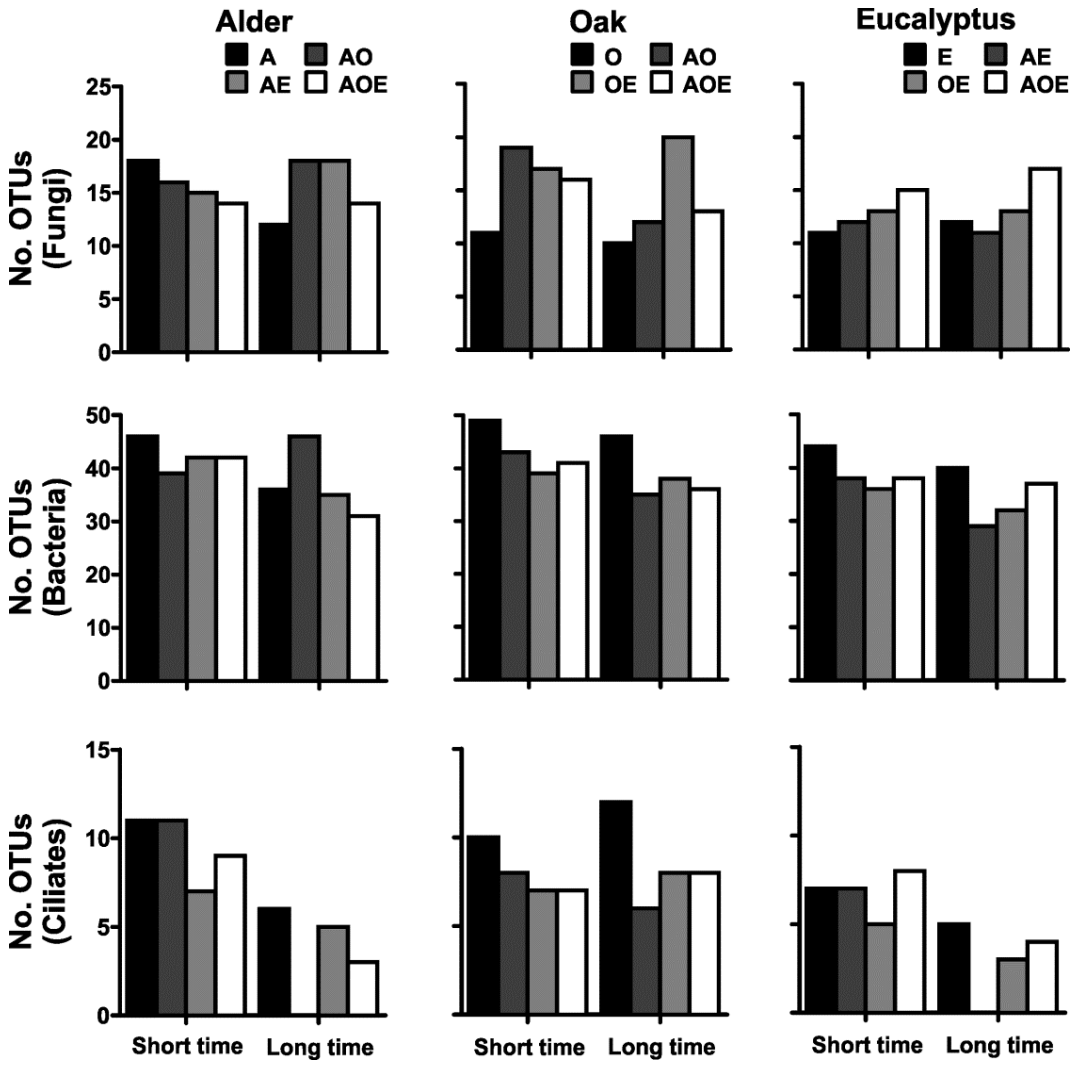

Figure 2.2 Number of OTUs from DGGE analyses of fungal, bacterial and ciliate assemblages associated with individual leaf species (A, *Alnus glutinosa*; O, *Quercus robur*; E, *Eucalyptus globulus*) from single- and mixed-leaf species treatments, after short (2 months) and long time (6 months) of leaf diversity loss.

2.3.2. Effects of plant litter diversity on leaf decomposition

Leaf mass loss varied between 26% in microcosms containing eucalyptus mixed with oak after long time of leaf diversity loss and 43% in microcosms with oak in mixtures with alder and eucalyptus at short time (Fig. 2.5). Decomposition of alder leaves was not affected by leaf species number or time after leaf species loss, but effects of leaf identity were marginally significant (3-way nested ANOVA, Table 2.1). However, the effects of leaf identity on decomposition of alder leaves became stronger after long time of leaf diversity loss (2-way nested ANOVA, P=0.005, F=11.59). Decomposition of alder leaves was higher in mixtures with oak than in

mixtures with eucalyptus or mixtures with oak and eucalyptus (Tukey-Kramer´s tests, P=0.024 and P=0.048, respectively). Decomposition of oak leaves was affected by leaf species number and interaction between species number and time after leaf species loss (3-way nested ANOVA, Table 2.1); leaf mass loss was higher in mixtures of 3 species than in treatments with oak alone (Tukey-Kramer´s test, P=0.040). Mass loss of eucalyptus leaves was affected by time after leaf diversity loss and marginally affected by leaf identity (3-way nested ANOVA, Table 2.1). Similarly to that found for alder leaves, the effects of leaf species identity on decomposition of eucalyptus leaves became stronger after long time of leaf diversity loss (2-way nested ANOVA, P=0.011, F=9.12). Mass loss of eucalyptus leaves was higher when eucalyptus was mixed with alder leaves than with oak leaves (Tukey-Kramer´s test, P=0.046).

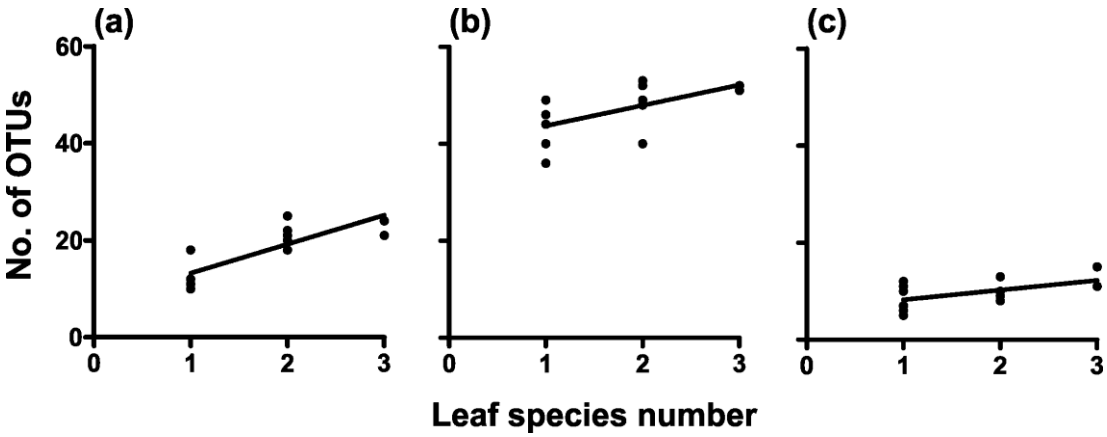

Figure 2.3 Relationship between number of OTUs of fungi (a), bacteria (b) and ciliates (c) and leaf species diversity. In mixtures of two and three leaf species, data represent total number of OTUs per leaf species treatment. Data were fitted to linear regressions. Fungi, Y=5.98X+7.25, r^2=0.69, P=0.0003; Bacteria, Y=4.21X+39.50, r^2=0.36, P=0.024; Ciliates, Y=1.99X+6.25, r^2=0.28, P=0.065.

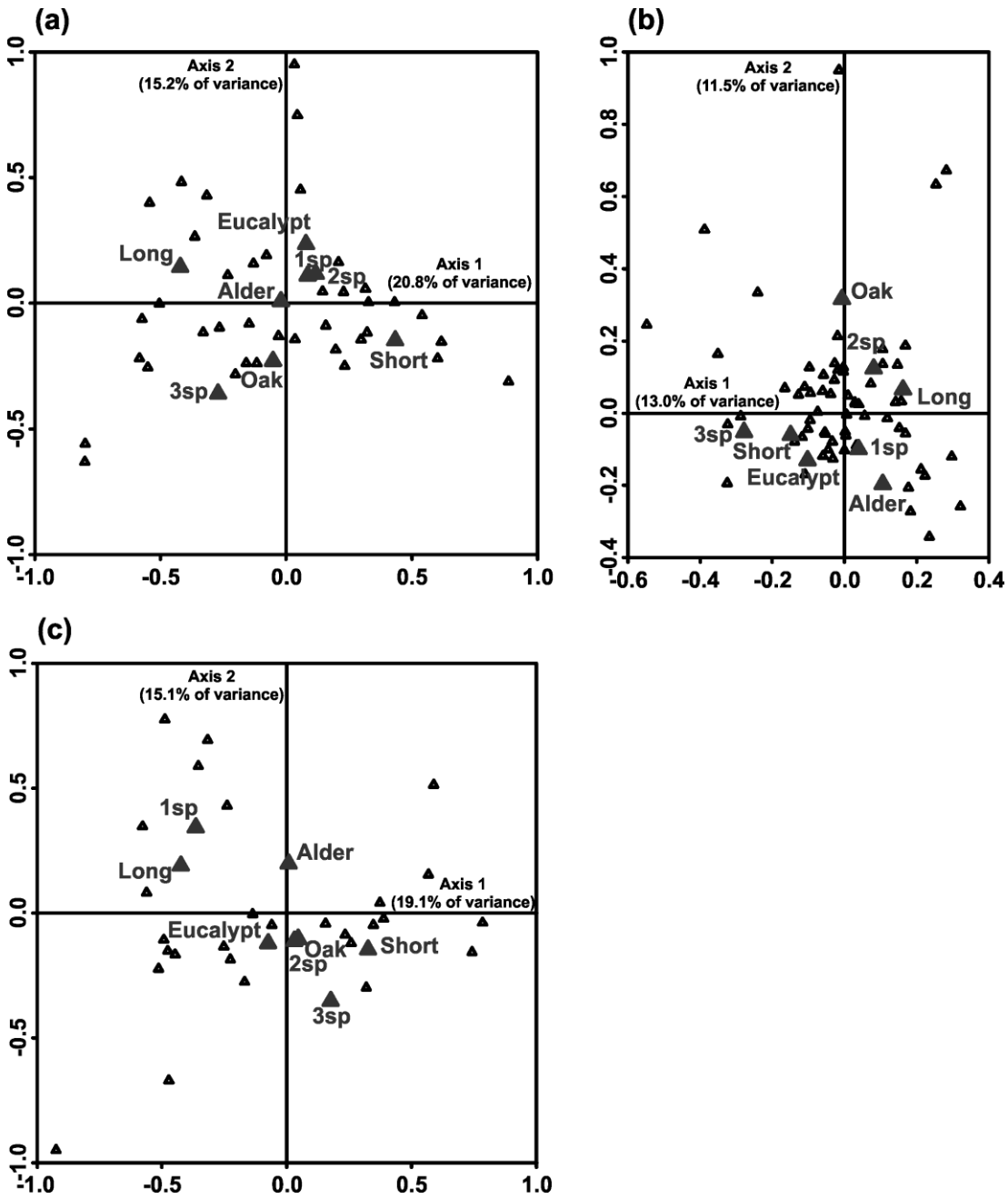

Figure 2.4 CA diagrams for ordination of fungal (a), bacterial (b) and ciliate (c) OTUs according to exposure time (short and long), leaf species number (1sp, 2sp and 3sp) and identity (alder, oak and eucalyptus).

Table 2.1 Effects of leaf species number (Sp nº), leaf species identity (ID), nested within leaf species number, and time after leaf diversity loss (T) on leaf mass loss and fungal biomass. In leaf mixtures, data came from individual leaf species.

Parameter	Leaf species	Factor	SS	DF	F	P
Leaf mass loss	Alder	Sp nº	26.7	2	0.47	0.630
		T	75.8	1	2.67	0.115
		Sp nº*T	20.5	2	0.36	0.701
		ID (Sp nº)	117.2	1	4.13	0.053
		ID (Sp nº)*T	52.1	1	1.84	0.188
		Error	680.7	24		
	Oak	Sp nº	115.0	2	3.77	0.038
		T	58.1	1	3.81	0.063
		Sp nº*T	122.7	2	4.02	0.031
		ID (Sp nº)	8.8	1	0.58	0.455
		ID (Sp nº)*T	14.0	1	0.91	0.349
		Error	366.4	24		
	Eucalyptus	Sp nº	26.4	2	0.63	0.539
		T	132.9	1	6.40	0.018
		Sp nº*T	90.1	2	2.17	0.136
		ID (Sp nº)	87.4	1	4.21	0.051
		ID (Sp nº)*T	33.2	1	1.60	0.218
		Error	498.9	24		
Fungal biomass	Alder	Sp nº	39497.4	2	6.75	0.007
		T	221.1	1	0.08	0.787
		Sp nº*T	11955.3	2	2.04	0.162
		ID (Sp nº)	4051.0	1	1.38	0.257
		ID (Sp nº)*T	3468.7	1	1.19	0.292
		Error	46814.2	16		
	Oak	Sp nº	73834.0	2	3.05	0.076
		T	94.0	1	0.01	0.931
		Sp nº*T	6609.0	2	0.27	0.765
		ID (Sp nº)	18124.0	1	1.50	0.239
		ID (Sp nº)*T	11212.0	1	0.93	0.350
		Error	193740.0	16		
	Eucalyptus	Sp nº	27038.0	2	10.90	0.001
		T	10665.5	1	8.60	0.010
		Sp nº*T	2211.0	2	0.89	0.430
		ID (Sp nº)	1000.1	1	0.81	0.383
		ID (Sp nº)*T	34.0	1	0.03	0.871
		Error	19851.3	16		

2.3.3. Effects of plant litter diversity on fungal biomass

Leaf-associated fungal biomass varied between 55 µg ergosterol g^{-1} leaf dry mass, in alder leaves in mixtures with eucalyptus after long time of leaf diversity loss, and 468 µg ergosterol g^{-1} leaf dry mass in oak leaves in mixtures with alder at the shorter time (Fig. 2.5). Fungal biomass on alder leaves was affected by leaf species number (3-way nested ANOVA, Table 2.1), with higher values in mixtures with three leaf species than with two leaf species (Tukey-Kramer´s test, P=0.024). Fungal biomass on oak leaves was not affected by leaf species number, leaf identity or time after leaf diversity loss (3-way nested ANOVA, Table 2.1). However, when effects of leaf diversity were analysed at the longer time after diversity loss, leaf species number affected fungal biomass on oak leaves (2-way nested ANOVA, P=0.017,

F=7.04). Moreover, fungal biomass on oak leaves decreased linearly with leaf species loss after long time (Linear regression, F=16.34, P=0.002, r^2=0.62, not shown). Fungal biomass associated with eucalyptus leaves was affected by leaf species number and time after leaf diversity loss (3-way nested ANOVA, Table 2.1), with overall higher biomass at the longer time (Tukey-Kramer´s test, P=0.006). The loss of leaf species led to a linear decrease in fungal biomass on eucalyptus leaves, with a stronger relationship at longer time (Linear regression, F=5.89, P=0.036, r^2=0.37, at short time; F=15.70, P=0.003, r^2=0.61, at long time; not shown).

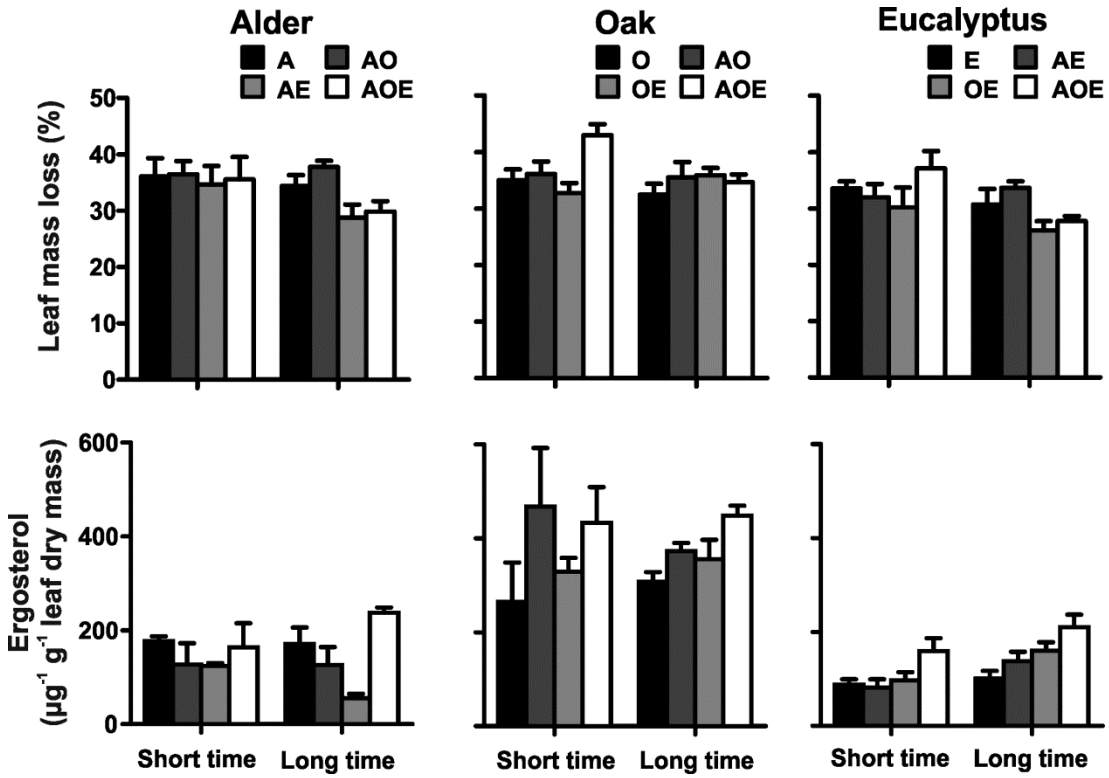

Figure 2.5 Percentage of leaf mass loss and fungal biomass from individual leaf species alone and in mixtures, after short (2 months) and long time (6 months) of leaf diversity loss. Values are mean + SEM; n=3 for fungal biomass and n=4 for leaf mass loss.

2.4. Discussion

Our study suggests that changes in plant species diversity of riparian corridors affect diversity and activity of microbes on decomposing plant litter in streams. The leaf species (alder, oak and eucalyptus) used in our study provide resources with different quality due to differences in their leaf C:N ratio. Thus, each leaf species might harbour different microbial assemblages that could provide inoculum to the different leaf species constituting the mixtures. Indeed, molecular diversity (as number of OTUs) of fungi on individual leaf species tended to be higher in leaf

species mixtures. However, this trend was not observed on bacterial and ciliate diversity, which was higher in single species treatments. Fungi are reported to have antagonistic interactions with bacteria during leaf decomposition (Mille-Lindblom & Tranvik, 2003), but fungi have morphological and physiological adaptations that allow them to colonize plant litter earlier than bacteria (Bärlocher, 1992), which might be outcompeted by fungi (Mille-Lindblom *et al.*, 2006). In addition, a reduction of bacterial diversity by the presence of fungi may decrease ciliate diversity because ciliates feed on bacteria and show preference for certain bacterial species (Dopheide *et al.*, 2011). This is consistent with the positive linear relationship between bacterial and ciliate diversity found in our study (not shown).

Although microbial assemblages on individual leaf species have shown different responses to leaf diversity loss, when microbial diversity as overall number of OTUs per leaf species treatment was considered, a positive relationship was found between leaf species diversity and the diversity of fungi and bacteria. A positive co-variation of fungal diversity with riparian plant species diversity was previously documented (Laitung & Chauvet, 2005; Lecerf *et al.*, 2005). Also, the replacement of native mixed forests by monocultures of eucalyptus in riparian corridors of streams in the Iberian Peninsula decreased the diversity of aquatic fungi with shifts in community composition (Bärlocher & Graça, 2002). In our study, the decrease in the number of OTUs per unit of leaf species lost was particularly high for fungi (6 OTUs/unit of leaf species diversity), pointing to a higher dependence of fungi than bacteria or ciliates on the diversity of plant litter resources. This agrees with the major role of fungi in early stages of plant-litter decomposition in streams (Pascoal & Cássio, 2004; Pascoal *et al.*, 2005); fungi have an efficient enzymatic machinery to degrade polysaccharides of plant cell walls, and their hyphae have high ability to penetrate substrates (Bärlocher, 1992). The relationship between fungal diversity and litter diversity found in our study (6 OTUs decrease per unit of leaf species lost) was even stronger than reported by others (1.7 fungal species decrease per unit of leaf species lost; Laitung & Chauvet, 2005). This apparent discrepancy might be related to differences in the levels of leaf litter diversity investigated (1-3 leaf species in our study versus 7-17 leaf species in Laitung & Chauvet, 2005). The positive relationship between the diversity of resources (litter) and the diversity of consumers (fungi) can be explained by mechanisms of niche differentiation (Tilman, 2000). We expected that more leaf species would provide a greater variety of resources that could allow the co-existence of more fungal species. However, it is conceivable that above a certain leaf diversity level, further increases in leaf diversity will not provide a proportional increase in nutrient supply or habitat structures for fungi. Therefore,

the dependence of fungal diversity on plant litter diversity is expected to be stronger at lower leaf diversity levels.

The shifts in the structure of microbial assemblages on decomposing leaves in response to plant species loss were accompanied by changes in decomposition of oak leaves, but not of alder or eucalyptus leaves. However, the identity of litter mixture affected leaf decomposition of alder and eucalyptus leaves, mainly after long time of leaf diversity loss. Actually, the composition of litter mixtures appears to have a greater role in leaf decomposition in streams than diversity of litter species (Swan & Palmer, 2004, 2006; Lecerf *et al.*, 2007) with the differences in litter quality explaining the effects of leaf identity on leaf decomposition (Lecerf & Chauvet, 2008; Schindler & Gessner, 2009; Fernandes *et al.*, 2012). In our study, decomposition of eucalyptus leaves (higher C:N ratio, lower quality) tended to be faster when mixed with alder (lower C:N ratio, higher quality) than with oak leaves (intermediate C:N ratio), suggesting that microbial assemblages on eucalyptus leaves might have benefited from the presence of compounds released by high quality leaves to fulfil their metabolic needs (Gessner *et al.*, 2010). Conversely, we found a deceleration of decomposition of alder leaves by the presence of eucalyptus at the longest time after leaf species loss. Also, fungal biomass on alder leaves was consistently lower when mixed with eucalyptus, especially at the longest time. Eucalyptus leaves contain oils and tannic acids that inhibit the growth of aquatic fungi (Canhoto & Graça, 1999). Thus, if inhibitory compounds were leached from eucalyptus leaves to the surrounding environment (Gessner *et al.*, 2010), microbial activity on other leaf species composing the mixture might also be inhibited.

In our study, the effects of leaf diversity were stronger on fungal biomass and diversity than on microbially-driven leaf decomposition. Moreover, fungal biomass and diversity tended to decrease as litter species were lost from the system, especially on oak and eucalyptus. This may have implications for freshwater invertebrates that preferentially feed on leaves colonized by microbes (Graça *et al.*, 2001; Duarte *et al.*, 2012). Moreover, fungal diversity correlates positively with leaf consumption rates by invertebrate shredders (Lecerf *et al.*, 2005). Thus, the effects of leaf diversity loss on fungal diversity and biomass observed in our study might have indirect impacts on plant-litter decomposition in streams.

Overall, leaf litter diversity and quality changed the structure of microbial assemblages and affected leaf decomposition and fungal biomass on individual litter species. Fungal biomass tended to decrease with leaf species loss, especially for lower quality leaf species (oak and eucalyptus) after long time of diversity loss. Leaf decomposition was mainly affected by leaf species identity at the longer time.

Microorganisms growing on low quality leaves appeared to benefit from the presence of other leaf species, as shown by higher fungal biomasses found in leaf mixtures. Conversely, the presence of eucalyptus lowered the decomposition of alder leaves at the longer time after leaf diversity loss. The eucalyptus species used in our study was introduced in the Iberian Peninsula almost two centuries ago, and nowadays vast areas are covered by monocultures of this exotic tree (Graça *et al.*, 2002). Alterations in diversity and quality of riparian vegetation can jeopardize litter inputs into streams with possible bottom-up effects to the functioning of detritus food-webs (Kominoski & Rosemond, 2012). Thus, protecting and/or restoring riparian vegetation is important to conserve microbial diversity and maintain the functioning of detritus food-webs in freshwaters.

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Chapter 3

Riparian plant diversity affects invertebrate shredder activity and resource quality to higher trophic levels in streams

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Riparian plant diversity affects invertebrate shredder activity and resource quality to higher trophic levels in streams

Submitted

Abstract

We used a pool of three plant species (alder, oak and eucalyptus) common in the riparian corridors of Iberian streams to test how plant litter diversity and time after diversity loss (2 and 6 months) affect leaf consumption, body elemental composition of invertebrate shredders and the quality of FPOM produced to other trophic levels. The number and identity of leaf species affected leaf consumption and FPOM production by invertebrate shredders. Time increased the positive diversity effects on leaf consumption and FPOM production. Changes in leaf quality altered C and N composition of invertebrate body suggesting that invertebrates can deviate from strict homeostasis. FPOM quality was positively correlated with leaf quality, but N percentage was higher in FPOM than in leaves. Leaf consumption by the animals decreased linearly with the increase in C:N imbalance between leaf litter and invertebrate body. Results suggest that changes in riparian plant diversity affect the activity of invertebrate shredders (leaf consumption and FPOM production), and the quality of food resources (FPOM and invertebrate shredders) to higher trophic levels in streams, and effects are likely to become stronger with time of plant diversity loss.

3.1. Introduction

The replacement of mixed forests by monocultures of tree species and degradation of riparian corridors by agriculture, industrial activities and urban settlements have led to a decrease of forest tree diversity throughout the world (Graça *et al.*, 2002; Foley *et al.*, 2005; Haines-Young, 2009). In low-order forested streams, allochthonous organic matter from riparian vegetation constitutes the main source of food and energy to aquatic biota (Vannote *et al.*, 1980). In these ecosystems, leaf-litter breakdown is a key ecological process mainly driven by microorganisms and invertebrates (Graça & Canhoto, 2006). Among microorganisms, aquatic fungi play a major role in leaf decomposition by producing an array of enzymes that soften leaf litter, making it more palatable for invertebrate shredders (Suberkropp, 1998). The activity of microorganisms and shredders on leaf litter transforms this coarse particulate organic matter (CPOM) into smaller particles. These, together with the spores released by fungi and the feces produced by invertebrates, constitute the fine particulate organic matter (FPOM) (Allan & Castillo, 2007), which can be consumed by the filter-feeders and gatherers (Cummins & Klug, 1979). However, little is known about the fate and nutritive value of FPOM comparing with CPOM in stream food webs (Allan & Castillo, 2007).

The role of individual nutrients in ecosystem processes is central in ecological stoichiometry because the balance of carbon (C) in relation to the key nutrients nitrogen (N) or phosphorus (P) in resources often limits the activity and growth of consumers. Generally, ecological stoichiometry predicts that heterotrophs are able to maintain elemental homeostasis (Sterner & Elser, 2002). However, elemental ratio of heterotrophs can shift when consuming a food source with a very high C:nutrient ratio compared with their body composition (DeMott *et al.*, 1998). Plant litter can have different chemical composition (Hladyz *et al.*, 2009; Schindler & Gessner, 2009), resulting in large stoichiometric imbalances between stream invertebrates and detritus that may alter leaf breakdown (Hladyz *et al.*, 2009) and invertebrate growth (Frost *et al.*, 2006). Microbial decomposers and invertebrate shredders preferentially feed on resources of high quality (low C:N), rich in labile compounds and nutrients (Canhoto & Graça, 1995; Fernandes *et al.*, 2012). Therefore, it is plausible that nutrient constraints due to changes in diversity or quality of leaf litter may result in alterations of elemental composition of invertebrates.

Effects of plant litter diversity on decomposition mediated by microorganisms (Fernandes *et al.*, 2012) and invertebrate shredders (Swan & Palmer, 2004, 2006a;

Lecerf *et al.*, 2007) appear to depend more on the composition of litter mixtures than on the number of litter species. Indeed, the decomposition of recalcitrant species in litter mixtures was slower than that predicted from individual litter species, due to preferential feeding of shredders on high-quality leaves (Swan & Palmer, 2006b). However, invertebrate shredders colonising diverse litter can also benefit from the stable substratum provided by refractory leaves, which may serve as habitat or to build their cases (Sanpera-Calbet *et al.*, 2009).

Given that leaf litter constitutes the base of detritus food webs in forested streams, changes in litter diversity may result in bottom-up effects for the entire food web (Kominoski *et al.*, 2010). The diversity of riparian plant species was positively correlated with fungal diversity (Laitung & Chauvet, 2005; Lecerf *et al.*, 2005) and this, in turn, was correlated with leaf consumption by an amphipod shredder (Lecerf *et al.*, 2005). Litter mixture effects on leaf decomposition by shredders may also result from indirect effects mediated by fungi due to a greater development of certain fungal species on leaf litter (Jabiol & Chauvet, 2012). Interestingly, the effects of plant litter diversity on leaf-associated microbes may change with time after biodiversity loss (Chapter 2), and this may alter the quality of plant litter (Wickings *et al.*, 2012) with possible consequences to invertebrates. This highlights the importance of incorporating longer time scales when examining the impacts of riparian plant biodiversity loss on detritus food webs.

We used a pool of three plant species (alder, oak and eucalyptus) common in the riparian corridors of Iberian streams to test how riparian plant diversity and time after diversity loss (2 and 6 months) affect leaf consumption, body elemental composition of invertebrate shredders and the quality of FPOM produced to other trophic levels. We expected that i) leaf consumption by invertebrate shredders would be superior in microcosms containing higher quality leaf litter, ii) the quality of FPOM produced would be positively correlated with the quality of leaf litter, iii) effects of litter diversity on leaf consumption would change with time after diversity loss, iv) body elemental composition of invertebrates would change with the quality of leaf litter, and finally, v) C:N imbalance between animal body and leaf litter would affect resource consumption by the invertebrates.

3.2. Methods

3.2.1. Leaf conditioning

Leaves of alder (*Alnus glutinosa* (L.) Gaertn.), oak (*Quercus robur* L.) and eucalyptus (*Eucalyptus globulus* Labill.) were collected from trees in October 2009,

immediately before abscission, and dried at room temperature. Leaves were soaked in deionised water and cut into disks (12 mm) with a cork borer. On 28 October 2009, sets of leaf disks with equal proportion of the 3 plant species were placed in fine-mesh bags (0.5-mm pore size) and immersed in the Estorãos stream, a mixed-forested stream in Portugal (41º78'19.4''N, 8º63'80.00''W), to allow microbial colonization. During leaf immersion, stream water had on average (±SEM) a temperature of 14 ± 1.0 ºC, a pH of 5.9 ± 0.06, a conductivity of 31 µS cm^{-1} and a redox potential of 51 ± 1.5 mV (Multiline F/set 3 no. 400327; WTW, Weilheim, Germany). Average (±SEM) nutrient concentrations in the stream water were: 0.30 ± 0.04 mg L^{-1} of $N\text{-}NO_3^-$, 0.003 ± 0.000 mg L^{-1} of $N\text{-}NO_2^-$, <0.01 mg L^{-1} of $N\text{-}NH_3$ and <0.003 $P\text{-}PO_4^{3-}$ (HACH kit, programs 351, 371, 385, and 490, respectively; HACH, Loveland, CO, USA).

After two weeks of stream immersion, mesh bags containing mixtures of alder, oak and eucalyptus leaves were brought to the laboratory. Simulation of leaf species loss was done in Erlenmeyer flasks with sterile stream water by placing sets of non-colonized leaf disks divided equally by 3, 2 or 1 leaf species plus 12 leaf disks (4 of each plant species) previously colonized in the stream to ensure microbial inoculum. This procedure was repeated every 30 days during 6 months by adding sets of non-colonized leaf disks, keeping leaf species proportion constant, and maintaining 12 leaf disks from the previous month as inoculum. Microcosms were kept under aeration and artificial light, at 16 ºC. Stream water was renewed every 15 days. After 2 and 6 months, leaf disks were used to assess the feeding behaviour of an invertebrate shredder, as described below.

3.2.2. Feeding experiment

Feeding experiments were carried out with early-stage larvae of the invertebrate shredder *Allogamus* sp., which is a common invertebrate genus in Iberian streams. Animals were collected and acclimated to the laboratory (2 weeks) and starved for 20 h prior to the experiment. Animals were placed in 250 mL Erlenmeyer flasks containing 100 mL of stream water and pre-weighed leaf disks treated as above. Leaf species treatments were: i) 18 leaf disks for 1 species treatment, ii) 9 leaf disks per species for 2 species treatments, and iii) 6 leaf disks per species for 3 species treatment. One animal was used per replicate (12 replicates per treatment). Flasks were kept under aeration with aquarium pumps, in a temperature-controlled room (16 °C), with artificial light (12h:12h photoperiod) for 8 days. Stream water was renewed every 4 days and fine particulate organic matter (FPOM) was collected. At

the end of the experiment, remaining leaf disks, FPOM and invertebrates were freeze-dried and stored at -80 ºC until used.

3.2.3. Invertebrate initial dry mass

The invertebrate case diameter (CD, mm) of each animal was measured to the nearest 0.01 mm under a stereomicroscope (Wild M8, Wild Heerbrugg, Heerbrugg, Switzerland) before the experiment and the animal dry mass (DM, mg) was estimated by linear regression as DM=2.9 x CD - 5.3 (r^2=0.64, P<0.05, n=40).

3.2.4. Leaf consumption and FPOM production

Leaf consumption was estimated as the difference between initial and final leaf mass weighed to the nearest 0.01 mg, and expressed per unit of invertebrate initial dry mass.

FPOM was collected by centrifugation (5000 rpm, 5 min; Centrifuge 5804R, Eppendorf, Madrid, Spain) and freeze-dried for 2 days before weighed to the nearest 0.01 mg. FPOM production was expressed as mass per unit of invertebrate initial dry mass.

3.2.5. Nutrient content

Freeze-dried samples of leaves, FPOM and invertebrate body were ground before elemental analysis. For each treatment, four replicates were pooled to achieve enough mass for nutrient analyses. Elements in invertebrates and FPOM (~1-1.5 mg) were analyzed in an elemental analyzer (CHNS-O EA-1108, Carlo Erba Instruments, ThermoScientific, Massachusetts, USA), using sulfanilamide as a standard, while elements in leaves (~100 mg) were analyzed in a LECO-CNS 2000 (LECO, St. Joseph, MI, USA), using EDTA as a standard. Analyses were done in the Centro de Apoio Científico e Tecnolóxico á Investigación (CACTI, University of Vigo, Spain). Total C and N were expressed as % of dry mass, and C:N ratio was expressed on a molar basis. C:N imbalance was calculated as the arithmetic difference in elemental ratios between invertebrate body and leaf litter (Cross *et al.*, 2003).

3.2.6. Statistical analyses

Three-way nested analyses of variance (ANOVA) were used to test if time after leaf diversity loss, leaf species diversity and identity (nested within leaf species diversity)

significantly affected leaf consumption and FPOM production. Because the design was unbalanced, we applied the Type III sum of squares using the Variance Estimation and Precision (VEPAC) module in Statistica 8.0 (Statsoft, Tulsa, OK, USA). Differences between treatments were analysed by the Tukey-Kramer's post-test, which is a modification of the Tukey's post-test for unbalanced designs (Zar, 2009).

Net diversity effects of leaf litter on leaf consumption and FPOM production were estimated as the difference between observed effects in leaf species mixtures and those expected based on the sum of effects of single leaf species weighted by their initial proportion in mixtures (Bärlocher & Corkum, 2003; Duarte *et al.*, 2006). The differences between observed and expected values were tested against the null hypothesis that the average difference equalled 0 (t-test).

The effects of time after leaf diversity loss and leaf species identity on FPOM quality and invertebrates body composition were analysed by two-way ANOVAs followed by Tukey's post-tests. Differences between final and initial invertebrate body composition (C, N and C:N ratio) were done by t-tests.

Linear regressions were used to establish the relationships between: i) FPOM production and leaf consumption; ii) elemental composition (C, N and C:N ratio) in leaves and in FPOM or animal body; and iii) C:N imbalance and leaf consumption. The effect of time after leaf diversity loss on the relationship between C:N imbalance and leaf consumption was tested by ANCOVA (Zar, 2009).

Analyses of variance were done in Statistica 8.0 for Windows (Statsoft, Inc., Tulsa, OK) and linear regressions, ANCOVA and t-tests were done in Prism 4.0 for Windows (GraphPad software Inc., San Diego, CA).

3.3. Results

3.3.1. Leaf consumption and FPOM production

Leaf consumption and FPOM production by invertebrate shredders was affected by leaf species diversity and identity, but not by the time after diversity loss (3 way-nested ANOVA, Table 3.1; Fig. 3.1). Leaf consumption and FPOM production were highest in mixtures with 3 leaf species and lowest for oak leaves alone (Tukey-Kramer´s test, $P<0.05$). A positive linear relationship was found between FPOM production and leaf consumption ($P<0.001$; $r^2=0.954$ and $r^2=0.933$, for short and long time after leaf diversity loss, respectively).

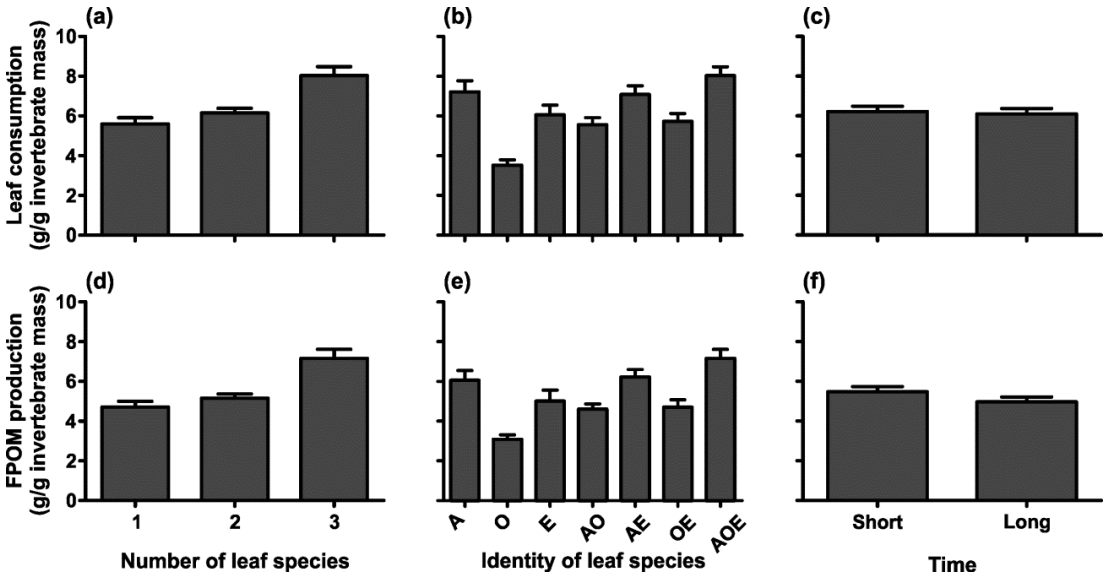

Figure 3.1 Leaf consumption (a, b and c) and FPOM production (d, e and f) by the invertebrate shredder after 8 days in microcosms. Invertebrates were fed on microbially-colonized leaf litter after short time (2 months) and long time (6 months) of simulation of leaf species loss. A, alder; O, oak; E, eucalyptus. M+SEM.

Table 3.1 Effects of leaf species number, leaf species identity (nested within species number) and time after leaf diversity loss on leaf consumption and FPOM production.

Parameter	Factor	SS	df	F	P
Leaf consumption	Nº of species	100.322	2	11.418	<0.001
	Identity (Nº of species)	203.393	4	11.574	<0.001
	Time	0.016	1	0.004	0.952
	Nº of species*Time	10.828	2	1.232	0.295
	Identity (Nº of species*Time)	20.393	4	1.160	0.331
	Error	654.578	149		
FPOM production	Nº of species	94.787	2	13.512	<0.001
	Identity (Nº of species)	144.918	4	10.329	<0.001
	Time	6.204	1	1.769	0.186
	Nº of species*Time	12.000	2	1.711	0.184
	Identity (Nº of species*Time)	30.720	4	2.190	0.073
	Error	498.053	142		

After short time of leaf diversity loss, positive net diversity effects were observed in mixtures of 3 leaf species because leaf consumption and FPOM production by the invertebrates were higher than expected based on data from individual leaf species (t-tests, P=0.027 and P=0.022, respectively; Fig. 3.2). However, no diversity effects were observed for mixtures of 2 leaf species (t-test, P>0.05). Time led to an increase of positive net diversity effects because significant effects were found not only in mixtures of 3 leaf species for leaf consumption and FPOM production (t-tests, P<0.001), but also on leaf consumption in mixtures of oak and eucalyptus (t-test, P=0.011), and on FPOM production in mixtures of alder and eucalyptus or oak and eucalyptus (t-tests, P=0.034 and P=0.008, respectively).

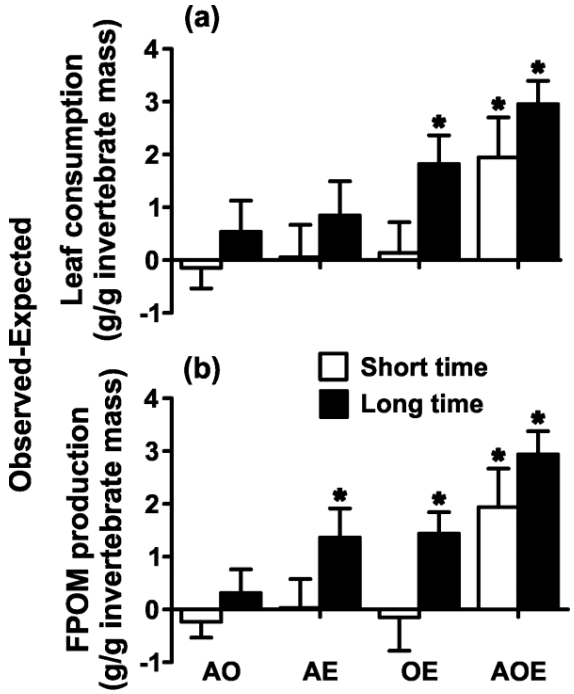

Figure 3.2 Net diversity effects of leaf litter on leaf consumption (a) and FPOM production (b) by the invertebrate shredder at short time (2 months) and long time (6 months) after leaf diversity loss. Net diversity effects were estimated as the difference between observed values in leaf mixtures and those expected based on the weighed sum of individual leaf species. Differences were tested against zero by a t-test; *P<0.05. A, alder; O, oak; E, eucalyptus. M+SEM.

3.3.2. FPOM quality

Percentage of C in FPOM differed with leaf species identity but not with time after leaf diversity loss (2-way ANOVA, Table 3.2; Fig. 3.3a). Percentage of C was highest in FPOM produced from eucalyptus leaves alone, and lowest in FPOM produced from oak leaves alone (Tukey's tests, P<0.05). A positive linear relationship was found between % of C in FPOM and % of C in leaves, at short and long time after leaf diversity loss (Table 3.3; Fig. 3.3b).

Percentage of N in FPOM varied with leaf identity but not with time after leaf diversity loss (2-way ANOVA, Table 3.2; Fig. 3.3c). Percentage of N in FPOM was highest in microcosms with alder leaves and lowest in microcosms with eucalyptus and oak leaves alone or in mixture (Tukey's tests, P<0.05). A positive linear relationship was found between % of N in FPOM and % of N in leaves, at short and long time after leaf diversity loss (Table 3.3; Fig. 3.3d). Percentage of N was always higher in FPOM than in leaves, and this difference was more pronounced in leaves with low % of N.

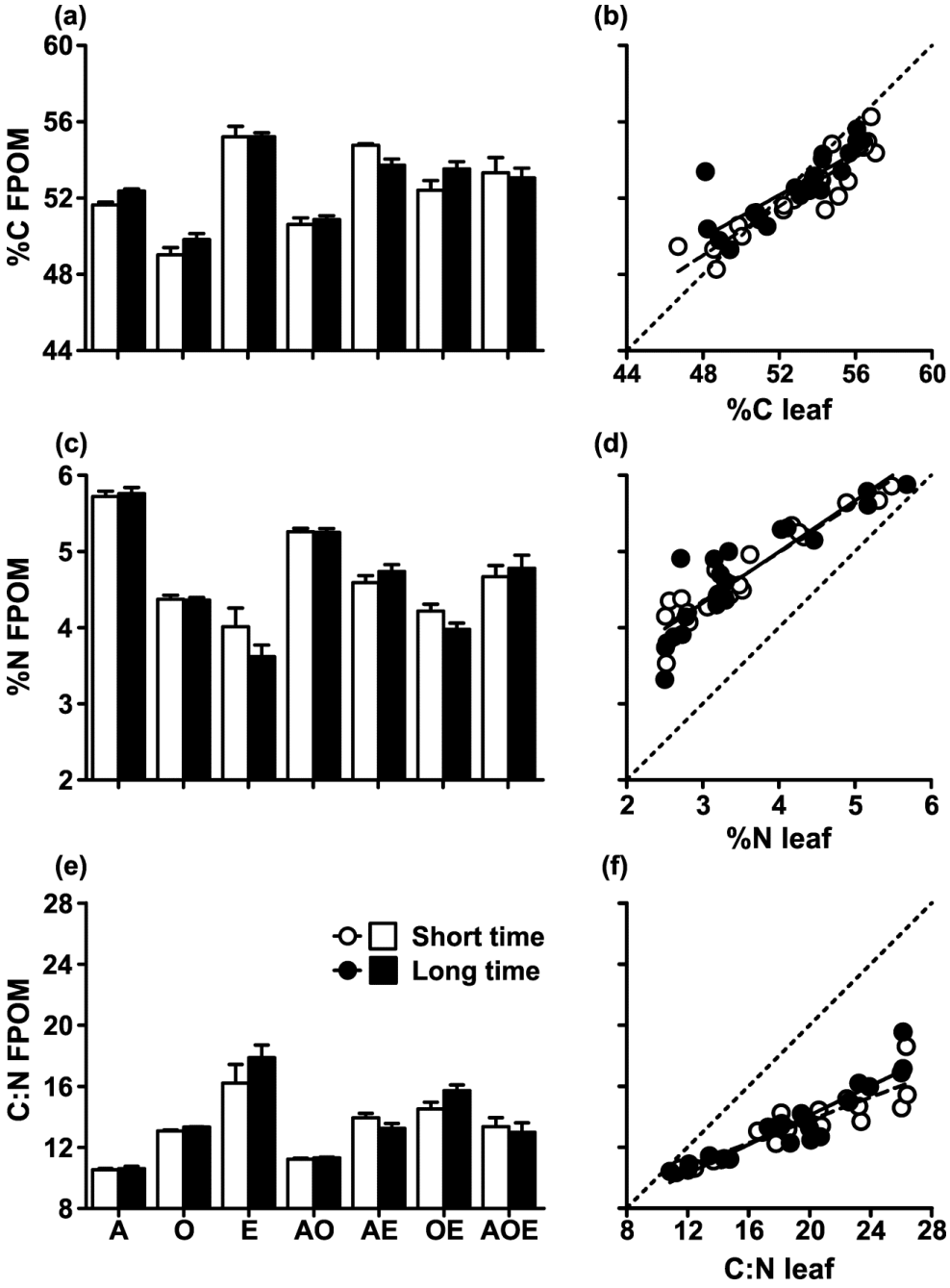

Figure 3.3 Percentage of C (a) and N (c), and C:N ratio (e) in the FPOM produced by the shredder during the feeding experiment at short time (2 months) and long time (6 months) after leaf diversity loss. Linear regressions between elemental composition of FPOM and leaf litter with respect to C (b), N (d) and C:N ratio (f). Dashed lines indicate 1:1 relationships. A, alder; O, oak; E, eucalyptus. M+SEM.

The C:N ratio of FPOM was only affected by leaf identity (2-way ANOVA, Table 3.2; Fig. 3.3e). C:N ratio was lowest in FPOM produced from alder or mixtures of alder and oak leaves, and was highest in FPOM from eucalyptus leaves alone (Tukey's tests, $P<0.05$). A positive linear relationship was found between FPOM C:N ratio and leaf C:N ratio (Table 3.3; Fig. 3.3f). C:N ratio was lower in FPOM than in leaves, especially for leaves with high C:N ratio.

Table 3.2 Effects of leaf species identity and time after leaf diversity loss on elemental composition of FPOM and invertebrate body, as carbon (C), nitrogen (N) and C:N ratio.

Parameter		Factor	SS	df	F	P
FPOM	C	Identity	143.30	6	49.95	<0.001
		Time	0.54	1	1.14	0.295
		Identity *Time	4.94	6	1.72	0.153
		Error	13.39	28		
	N	Identity	15.87	6	66.35	<0.001
		Time	0.03	1	0.70	0.411
		Identity *Time	0.34	6	1.42	0.242
		Error	1.12	28		
	C:N	Identity	127.30	6	38.69	<0.001
		Time	0.77	1	1.41	0.246
		Identity *Time	4.58	6	1.39	0.252
		Error	15.36	28		
Invertebrate body	C	Identity	22.49	6	8.80	<0.001
		Time	9.67	1	22.69	<0.001
		Identity *Time	3.68	6	1.44	0.235
		Error	11.93	28		
	N	Identity	2.15	6	2.69	0.034
		Time	1.04	1	7.82	0.009
		Identity *Time	1.11	6	1.40	0.251
		Error	3.72	28		
	C:N	Identity	1.67	6	4.19	0.004
		Time	0.82	1	12.28	0.002
		Identity *Time	0.60	6	1.51	0.211
		Error	1.86	28		

3.3.3. Invertebrate body elemental composition

Percentage of C in the invertebrate body was affected by leaf identity and time (2-way ANOVA, Table 3.2; Fig. 3.4a), with higher values at longer time after leaf diversity loss (Tukey's test, $P<0.05$). The % of C was highest in invertebrates that fed on mixtures of alder and eucalyptus leaves, and was lowest in invertebrates that fed on oak leaves alone (Tukey's tests, $P<0.05$). The % of C in invertebrate body increased in treatments with leaf mixtures containing eucalytus for both times (t-test, $P<0.05$) and in single treatments with alder or eucalyptus after long time (t-test, $P=0.017$ and $P=0.004$, respectively). Moreover, % of C in invertebrate body increased linearly with the increase in % of C in leaves (Table 3.3; Fig. 3.4b).

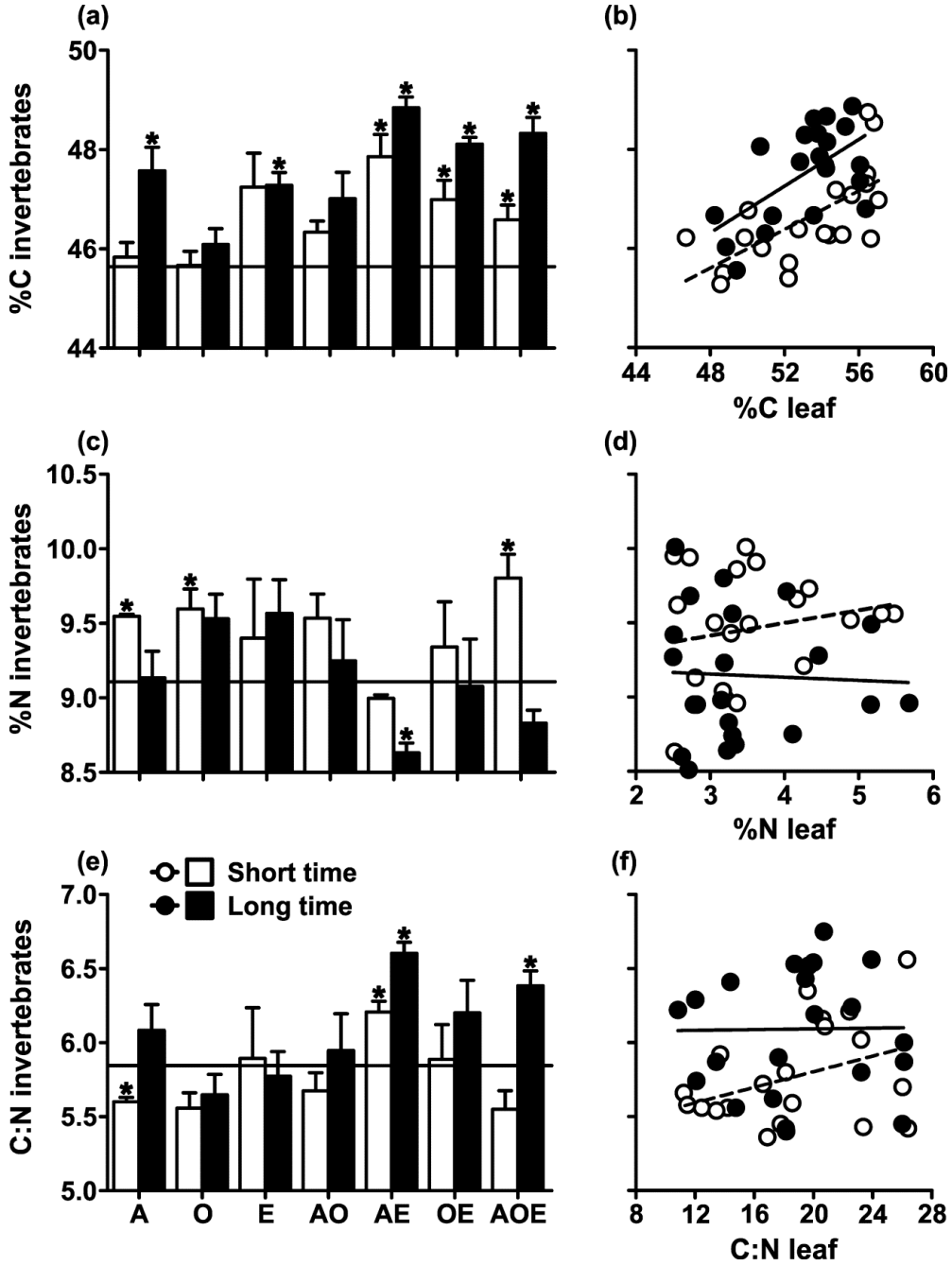

Figure 3.4 Percentage of C (a) and N (c), and C:N ratio (e) in the invertebrate body after the feeding experiment at short time (2 months) and long time (6 months) after leaf diversity loss. Linear regressions between elemental composition of animal body and leaf litter with respect to C (b), N (d) and C:N ratio (f). Horizontal lines (a, c, e) indicate initial elemental composition of animal body before feeding. *, indicates significant differences in elemental composition of animal body before and after the feeding experiment (t-test). A, alder; O, oak; E, eucalyptus. M+SEM.

Percentage of N in invertebrate body was affected by leaf identity and time (2-way ANOVA, Table 3.2; Fig. 3.4c), with lower values at long time after leaf diversity loss (Tukey's test, $P<0.05$). Invertebrates had the lowest % of N when fed on mixtures of alder and eucalyptus leaves (Tukey's test, $P<0.05$). Compared with the initial body composition, % of N in invertebrates increased in treatments with alder or oak

leaves alone (t-test, P=0.005 and P=0.033, respectively) and in mixtures with 3 leaf species after short time of leaf diversity loss (t-test, P=0.017). Conversely, the % of N decreased in animals that fed on mixtures of alder and eucalyptus leaves after long time of leaf diversity loss (t-test, P=0.009). No relationship was found between the % of N in invertebrate body and the % of N in leaves (Table 3.3, Fig. 3.4d).

The C:N ratio in invertebrate body was also affected by leaf identity and time (2-way ANOVA, Table 3.2, Fig. 3.4e), with higher values at long time after leaf diversity loss (Tukey's test, P<0.05). The highest C:N ratios were found in invertebrates that were fed on mixtures of alder and eucalyptus leaves (Tukey's test, P<0.05). Compared with the initial invertebrate elemental composition, the C:N ratio increased in animals fed on mixtures of alder and eucalyptus leaves for both times, and on mixtures of 3 leaf species at the longer time (t-test, P<0.05). Conversely, invertebrates that were fed on alder leaves after short time of leaf diversity loss had lower C:N ratio than at the beginning of the experiment (t-test, P=0.022). No relationship was found between invertebrate C:N ratio and initial C:N ratio of leaves (Table 3.3, Fig. 3.4f).

Table 3.3 Linear regressions of the relationship between elemental composition of leaf litter and FPOM or invertebrate body at short and long time after leaf diversity loss (2 and 6 months, respectively).

Time		Equation	P	r^2
Leaf litter against FPOM				
Short	%C	Y=0.642X+18.16	<0.01	0.84
Long		Y=0.555X+23.29	<0.01	0.66
Short	%N	Y=0.642X+2.418	<0.01	0.89
Long		Y=0.673X+2.305	<0.01	0.80
Short	C:N	Y=0.376X+6.260	<0.01	0.82
Long		Y=0.481X+4.491	<0.01	0.86
Leaf litter against invertebrate body				
Short	%C	Y=0.195X+36.25	<0.01	0.43
Long		Y=0.235X+35.02	<0.01	0.36
Short	%N	Y=0.085X+9.157	0.40	0.04
Long		Y=-0.021X+9.219	0.84	<0.01
Short	C:N	Y=0.026X+5.276	0.10	0.14
Long		Y=0.001X+6.067	0.95	<0.01

3.3.4. C:N imbalance and leaf consumption

C:N imbalances between leaf litter and invertebrate body ranged from 6 in alder to 20 in eucalyptus leaves (Table 3.4). Leaf consumption by the shredder decreased linearly with increasing C:N imbalance (P<0.001; Fig. 3.5), but the relationship did not change with time after leaf diversity loss (ANCOVA, F=0.83, P=0.36). Leaf consumption by the shredder decreased from approximately 45% to 14% as C:N imbalance increased from 6 to 20, corresponding to a 2% decrease in leaf consumption per unit of C:N imbalance increase.

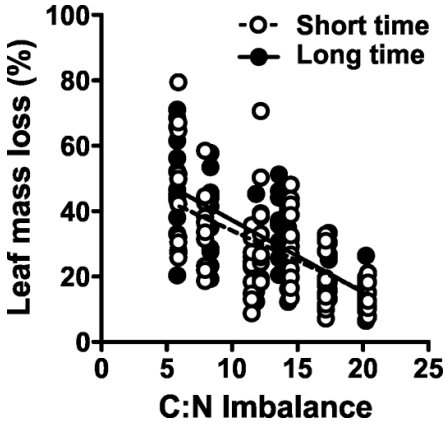

Figure 3.5 Linear regression of leaf consumption against C:N imbalance between invertebrate body (consumer) and leaf litter (resource), at short time (2 months) and long time (6 months) after leaf diversity loss. Short time, Y=-1.84X+52.42; r^2=0.33; P<0.0001. Long time, Y=-2.20X+59.24; r^2=0.46; P<0.0001.

Table 3.4 Elemental composition of leaf litter used to feed invertebrates and C:N imbalance between leaf litter and invertebrate body. Leaf litter was colonized in a stream and then transferred to microcosms to simulate leaf species loss at short time (2 months) and long time (6 months). Nitrogen (N) and carbon (C) are percentage of dry mass and C:N is expressed as molar ratio. A, alder; O, oak; E, eucalyptus. M±SE.

Leaf treatment	Time	Leaf C (%)	Leaf N (%)	Leaf C:N	C:N imbalance
A	Short	52.41±0.18	5.23±0.18	11.73±0.37	5.88±0.37
	Long	53.17±0.22	5.34±0.17	11.65±0.40	5.80±0.40
O	Short	47.98±0.65	3.23±0.09	17.35±0.61	11.50±0.61
	Long	48.83±0.34	3.22±0.04	17.67±0.25	11.83±0.25
E	Short	56.83±0.12	2.53±0.02	26.24±0.12	20.39±0.12
	Long	56.17±0.10	2.51±0.01	26.08±0.04	20.23±0.04
AO	Short	50.23±0.28	4.25±0.05	13.78±0.23	7.94±0.23
	Long	51.00±0.19	4.20±0.13	14.19±0.39	8.35±0.39
AE	Short	56.44±0.02	3.24±0.06	20.32±0.37	14.47±0.37
	Long	53.02±2.45	3.08±0.19	20.12±0.30	14.27±0.30
OE	Short	54.75±0.43	2.78±0.03	23.00±0.29	17.16±0.29
	Long	53.98±0.15	2.71±0.05	23.24±0.38	17.39±0.38
AOE	Short	54.68±0.28	3.54±0.04	18.02±0.12	12.18±0.12
	Long	54.01±0.21	3.25±0.05	19.42±0.39	13.57±0.39

3.4. Discussion

Our results suggested that the riparian plant diversity affect litter consumption and FPOM production by stream invertebrate shredders, and effects become stronger at the longer time after plant diversity loss. Positive net diversity effects were observed in mixtures of three leaf species at short time, while at longer time positive net diversity effects also emerged in mixtures of two leaf species (eucalyptus with oak or alder). This agrees with that reported in a recent meta-analysis which brought to the forefront that the frequency of synergistic diversity effects of riparian plant litter on

carbon and nutrient dynamics in streams tend to increase with time (Lecerf *et al.*, 2011).

In our study, plant diversity effects on leaf consumption by invertebrates might have resulted from changes in leaf litter identity/quality. In fact, changes in litter diversity led to changes in C:N imbalances between litter and invertebrates that ranged from 6 in alder leaves to 20 in eucalyptus leaves, with intermediate values for leaf mixtures. The C:N imbalances found in our study were within the lower range reported by others (19-21, Lauridsen *et al.*, 2012; 66-75, Cross *et al.*, 2003; 8-107, Hladyz *et al.*, 2009). This was not unexpected because in our study leaf litter given to invertebrates was colonized by microbes, which might have decreased the C:N ratio of leaves comparing to their initial values. Similarly to that observed by Hladyz and collaborators (2009), a negative linear relationship was found between C:N imbalances and leaf mass loss. Values for C:N imbalance of leaf mixture with the highest diversity fell in the middle of the observed imbalance range, i.e. the loss of leaf diversity may result in higher or lower resource quality for invertebrate shredders and explain the respective increase or decrease of leaf mass loss. These findings suggest that changes in riparian plant diversity alter consumer-resource imbalances resulting in changes of stream ecosystem processes.

The imbalance between body elemental composition of shredders and their food resources (detritus) are the highest among invertebrates in streams (Cross *et al.*, 2003; Evans-White *et al.*, 2005). This suggests that shredders can feed on a wide variety of detritus with different elemental composition. In our study, invertebrate shredders preferentially fed on alder leaves as found by others (Canhoto & Graça, 1995; Canhoto & Graça, 1996), probably because these leaves had high quality (low C:N ratio) minimizing nutrient imbalance. Surprisingly, the consumption of leaves with the lowest quality (eucalyptus with high C:N ratio) was considerably high, especially at short time after diversity loss. The high invertebrate consumption of lower quality leaf litter might occur as a compensatory behaviour to fulfil the need for specific nutrients (Iversen, 1974; Friberg & Jacobsen, 1999; Albariño & Balseiro, 2001), probably in an attempt to maintain elemental homeostasis (Sterner & Elser, 2002). However, in our study, invertebrate body elemental composition changed with the quality of food resources, especially in terms of C, and also varied with time after leaf diversity loss. Under high C:N imbalances between leaf litter and invertebrates, animals may control the excess of C by regulating the uptake of C-rich compounds across the gut, excreting organic C, or increasing metabolic activity and respiration along with CO_2 release (Hessen & Anderson, 2008). However, in our study none of these mechanisms seemed to be used by the animals to cope with

increasing C:N imbalances because the % of C in invertebrates increased with increasing % of C in leaf litter. Surplus of C can sometimes be used for fitness-promoting purposes, such as structural defences, protective metabolites, heat gain or energy storage (Hessen & Anderson, 2008), which might have occurred in our study. Besides changes in % of C, some leaf treatments led to changes in % of N suggesting that invertebrates can deviate from strict homeostasis. Actually, changes in consumer body composition due to changes in resource quality were previously reported. For instance, in P enriched streams, P content in leaf litter, epilithon and consumer invertebrates increased (Small & Pringle, 2010). Also, a reduction in riparian vegetation canopy led to a decrease in terrestrial detritus inputs and to an increase in macrophytes and filamentous algae, which have higher nutritional quality (lower C:N ratio) than plant litter, resulting in a decrease of the C:N ratio in the freshwater omnivorous crayfish *Cherax destructor* (Giling *et al.*, 2012). Therefore, changes in resources may have direct effects on invertebrate body composition with implications to other invertebrates and fishes feeding on them.

Feeding activity of invertebrate shredders by fragmenting larger particles into smaller ones and through the production of feces leads to FPOM release (Allan & Castillo, 2007). Therefore, changes in litter quality are expected to alter the quality of FPOM available for other aquatic organisms. In our study, FPOM quality was positively correlated with leaf litter quality although FPOM had higher % of N. In addition, FPOM produced from the feeding activity on leaves with lower initial % of N presented proportionally higher % of N. Some leaf species may have N compounds that are more recalcitrant and, consequently, more difficult to digest and assimilate, resulting in higher % of N in feces (Balseiro & Albariño, 2006) or in overall FPOM (present study). This suggests that changes in riparian vegetation may affect the quality of FPOM available to the filter-feeders and gatherers, with implications to aquatic detritus food webs.

Overall, we found that changes in leaf diversity (both number and identity of leaf species) affected leaf consumption and FPOM production by invertebrate shredders. Leaf diversity effects became stronger at the longer time because positive effects were more frequent after long time of plant biodiversity loss. Changes in leaf identity were translated into changes in C:N imbalances between invertebrate shredders and their resources, with leaf consumption decreasing with the increase in C:N imbalance. Leaf diversity altered invertebrate body elemental composition and FPOM quality with possible implications to other aquatic invertebrates and fishes. Therefore, our results support that changes in riparian plant diversity may affect detritus food webs directly through changes in the activity of invertebrate shredders

(leaf consumption and FPOM production) and indirectly through alterations in the quality of food resources (FPOM and invertebrates shredders) to higher trophic levels in streams.

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Chapter 4

Eutrophication modulates diversity effects on leaf-litter decomposition in streams

Abstract

Although freshwater ecosystems are severely impacted by changes in riparian vegetation and increasing eutrophication, their interactive effects on litter decomposition and associated biota in streams remains poorly understood. In this study, 5 leaf species were placed in coarse-mesh bags alone or in mixtures and immersed in six low-order streams along an eutrophication gradient. Leaf mass loss, and fungal and invertebrate biomasses on leaves varied with the leaf species identity. Invertebrate biomass was not affected by leaf species number, but fungal biomass was higher in mixtures with 5 leaf species. Leaf mass loss was higher in leaf mixtures than in single leaf species. Higher N immobilization occurred in moderately and highly eutrophic streams comparing to the most oligotrophic one and in leaves with the lowest initial N concentration. Apart from the most eutrophic stream, a positive linear relationship between initial N concentration in leaves and leaf mass loss was found, and the slopes increased with increasing eutrophication, suggesting that the effects of litter quality might increase in eutrophic strems. Leaf-litter decomposition in mixtures was higher than that expected based on the sum of decomposition of individual leaf species (positive diversity effects), but these effects were only evident in the most oligotrophic streams. Overall results suggest that eutrophication may modulate leaf diversity effects on leaf decomposition in streams. It is conceivable that if water quality of eutrophic streams is improved, a strengthening of positive leaf diversity effects on litter decomposition in streams may occur in the near future.

4.1. Introduction

Aquatic ecosystems are particularly vulnerable to global change and freshwaters are currently among the most endangered ecosystems in the world (Malmqvist & Rundle, 2002; Dudgeon *et al.*, 2006). Anthropogenic activities have conducted to a severe degradation of water resources, and water pollution was identified as a major threat to human water security and river biodiversity (Vörösmarty *et al.*, 2010). Nutrient loading come out as a dominating source of pollution (Vörösmarty *et al.*, 2010), which has been increasing in rivers over the past century (Malmqvist & Rundle, 2002). Besides water pollution, changes in land use, mainly due to intensification of agriculture and expansion of urban settlements, are greatly degrading forests throughout the world by altering species composition or decreasing plant diversity in riparian corridors (Graça *et al.*, 2002; Foley *et al.*, 2005; Haines-Young, 2009).

In headwater-forested streams, plant detritus from riparian vegetation is the main source of food and energy to aquatic biota (Vannote *et al.*, 1980). In these ecosystems, leaf-litter decomposition is a key process driven by microbes, mainly fungi, and invertebrates (Graça & Canhoto, 2006; Gessner *et al.*, 2007). A recent study covering 100 European streams showed that leaf decomposition was stimulated by increasing nutrient concentration until a certain level above which the process became inhibited (Woodward *et al.*, 2012). On the other hand, changes in riparian plant diversity may result in alterations in the quantity or quality of litter inputs to streams (Webster *et al.*, 1990). This may affect the food resources available to aquatic biota dependent on plant detritus (Webster *et al.*, 1990; Pozo *et al.*, 1997) leading to shifts in the structure of aquatic communities (Bärlocher & Graça, 2002; Kominoski & Pringle, 2009) and/or leaf decomposition rates (Kominoski & Pringle, 2009; Kominoski *et al.*, 2011). A compilation of manipulative studies on the effects of leaf-litter diversity on leaf decomposition showed that 44% of litter mixtures decomposed faster than predicted from the sum of single litter species (synergistic effects) and 39% of litter mixtures decomposed slower than expected from individual species decomposition (antagonistic effects, Lecerf *et al.*, 2011). Moreover, environmental context might change the magnitude or direction of leaf diversity effects. For instance, antagonistic effects of litter diversity on leaf decomposition were found in summer but not in autumn (Swan & Palmer, 2004), and the antagonistic effects were also suppressed by nutrient enrichment in the stream water (Rosemond *et al.*, 2010). Although changes in riparian diversity and nutrient concentrations in streams may occur simultaneously, their interactive effects

on leaf decomposition and associated aquatic biota remain poorly understood (but see, Rosemond *et al.*, 2010).

In this study, we assessed the effects of riparian plant diversity (species quality and number) and eutrophication on leaf-litter decomposition and the associated decomposer communities in streams. For that, leaves of five plant species common in the riparian area of the study sites (alder, chestnut, eucalyptus, plane tree and oak) were enclosed alone or in mixtures in coarse mesh bags and immersed in six streams along an eutrophication gradient. We tested: i) if leaf litter decomposition and decomposer activity depended more on plant species number or quality; ii) if putative diversity effects could be predicted by comparing leaf decomposition in plant mixtures with that expected from the weighted sum of individual plant species (additive model); and iii) how eutrophication would modulate leaf diversity effects. Leaf litter diversity effects on litter decomposition were expected to be positive because highly diverse litter mixtures would provide more diverse resources and a more stable habitat to microbial decomposers and invertebrate detritivores. Because microbes can obtain nutrients from both leaf litter and stream water (Suberkropp & Chauvet, 1995), eutrophication was expected to affect litter-associated microbial activity and alter litter nutrient content, and consequently its palatability to invertebrate detritivorus, potentially attenuating positive diversity effects of leaf litter.

4.2. Methods

4.2.1. Study sites

Field experiments were conducted at six stream sites of the Ave River basin (Northwest of Portugal). The Agra Stream is near the spring of the Ave River (Serra da Cabreira) in an area with low human influence. Riparian vegetation was dominated by *Castanea sativa* Mill. and *Quercus* sp. and the stream substrate was composed by boulders and pebbles. Oliveira, Andorinhas and Agrela streams are in an area with some agricultural activities. In the Oliveira Stream, the riparian vegetation was composed by *Alnus glutinosa* (L.) Gaertn., *Quercus* sp., *Platanus* sp. and *C. sativa*, and the streambed was constituted by boulders, pebbles and gravel. The Andorinhas Stream was bordered by *A. glutinosa*, *Quercus* sp. and *C. sativa* and the streambed was composed by sand and gravel. The Agrela Stream was bordered by *A. glutinosa*, *Quercus* sp. and *Eucalyptus globulus* Labill.; sand and silt dominated the substrate and boulders were also present. The Selho River is near the city of Guimarães. At the study site, the stream was bordered by *Populus* sp. and *A. glutinosa*, and the substrate was constituted by sand, gravel and boulders.

The Couros Stream crosses the city of Guimarães and, at the study site, was bordered by agricultural fields and occasionally by *Populus* sp.; the streambed was dominated by sand.

4.2.2. Experimental setup

Leaves of *A. glutinosa* (alder, A), *C. sativa* (chestnut, C), *Platanus* sp. (plane tree, P), *Quercus robur* L. (oak, O) and *E. globulus* (eucalyptus, E) were collected just before abscission in autumn 2009, air-dried and stored until used. Leaf species were placed in plastic mesh bags (5-mm mesh; 30 x 23 cm) alone (A, C, P, O, E) or in selected combinations of two species (A+C, A+E, A+O), three species (A+C+O, A+E+P, A+E+O) and five species (All), in a total of 12 treatments (four replicates per treatment). Leaves were weighed in groups of 4 ± 0.001 g. In mixtures, leaf mass was proportionally divided by the number of leaf species in each treatment (2 g, 1.33 g and 0.8 g for mixtures of two, three and five species, respectively). Leaf bags were immersed at each stream site on 10th November 2010. After 38 days, leaf bags were retrieved, placed individually in plastic bags, and transported in a cool box (4 ºC) to the laboratory. In the laboratory, leaf litter was removed from each bag and rinsed with tap water over an 850-µm mesh sieve to retain invertebrates. Leaf material was cut into 12-mm leaf disks and used to estimate fungal biomass and induce fungal sporulation. The remaining leaf material was used to estimate leaf mass loss and nutrient concentration in leaves.

4.2.3. Physical and chemical analyses of the stream water

Physical and chemical parameters of the stream water were measured for 4 times during the study period at each sampling site. Conductivity, dissolved oxygen, pH and temperature were measured *in situ* with field probes (Multiline F/set 3 no. 400327, WTW). Stream water samples were collected in plastic bottles, transported in a cool box and used (within 24h) for chemical analyses. Concentrations of $N\text{-}NO_3$ (HACH kit, cadmium reduction method, LR), $N\text{-}NO_2$ (HACH kit, diazotization method, LR), $N\text{-}NH_3$ (HACH kit, salicylate method) and $P\text{-}PO_4$ (HACH kit, ascorbic acid method) in stream water were measured using a HACH DR/2000 photometer (Hach company, Loveland, CO, USA) according to the manufacturer instructions.

4.2.4. Identification of fungal spores and quantification of mycelial biomass

Fungal sporulation was induced by aeration of five leaf disks from each replicate bag in 75 mL of filtered stream water for 48 ± 4 h, at 16 ºC. Appropriate aliquots of

conidial suspensions were filtered (0.45-µm pore size, Millipore), and conidia were stained with 0.05% cotton blue in lactic acid. At least 300 spores per filter were identified and counted under a light microscope to determine the contribution of each aquatic hyphomycete species to the total conidial production in assemblages. Fungal sporulation rates were calculated for each species as the number of spores released per gram of leaf dry mass per day.

Mycelial biomass was estimated from ergosterol concentration on leaves (Gessner, 2005). Lipids were extracted from sets of five leaf disks by heating (80 °C, 30 min) in 8 g L^{-1} KOH in methanol, purified by solid-phase extraction and eluted in isopropanol. Ergosterol was quantified by high performance liquid chromatography (Beckmann Gold System) using a LiChrospher RP18 column (250×4 mm, Merck). The system was run isocratically with HPLC-grade methanol at 1.4 mL min^{-1} and 33 °C. Ergosterol was detected at 282 nm and quantified based on a standard curve of ergosterol in isopropanol (Sigma). Ergosterol concentration was converted to fungal biomass assuming 5.5 µg ergosterol mg^{-1} mycelial dry mass (Gessner & Chauvet, 1993).

4.2.5. Invertebrate identification and biomass

Leaf-associated invertebrates were preserved in 96% ethanol and identified to the lowest possible taxonomic level (Tachet *et al.*, 2010). After identification and counting, invertebrates were dried to constant mass (72 ± 24h) and weighed to the nearest ±0.0001 g.

4.2.6. Leaf mass loss

The remaining leaf litter was freeze-dried to constant mass (72 ± 24h) and weighed (±0.0001 g). Leaf mass loss was estimated as the difference between leaf mass at the beginning and at the end of experiment. Additional leaf disks from the 12 treatments were freeze-dried to constant mass and weighed to determine the conversion factor between air-dried mass and freeze-dried mass of leaves.

4.2.7. Nitrogen concentration in leaves

Portions of leaf material (ca. 120 mg) were ground and used to estimate initial and final concentration of nitrogen (N) in each leaf litter treatment. Nitrogen concentration in leaf litter was determined in a LECO-CNS 2000 Elemental Analyzer (Leco Corp., St. Joseph, MI, USA) at the Centro de Apoio Científico e Tecnolóxico á Investigación (CACTI, University of Vigo, Spain).

4.2.8. Statistical analyses

A principal component analysis (PCA) was used to ordinate sites according to nutrient concentrations in the stream water (CANOCO version 4.5, Microcomputer Power, Ithaca, New York).

Nested ANOVAs were used to test the effects of leaf species number, identity (nested within species number) and stream eutrophication level on leaf mass loss, fungal biomass and invertebrate biomass (Zar, 2009). Because the experimental design was unbalanced, we applied Type III analyses of variance using the Variance Estimation and Precision (VEPAC) module in STATISTICA 8.0 (Statsoft, Tulsa, OK, USA). Differences between treatments were analysed using the Tukey-Krammer´s post-test (Zar, 2009).

Leaf diversity effects on leaf decomposition were further assessed as deviation from additivity, i.e. as the difference between observed leaf mass loss in mixtures and expected values based on the sum of individual leaf species mass loss weighed by their proportion in the mixture (Duarte *et al.*, 2006). Differences between observed and expected leaf mass loss were tested against the null hypothesis that the average difference equalled zero (t-test) (Duarte *et al.*, 2006). Comparisons were done at each diversity level using average data of all streams or at each stream using average data of all diversity levels.

Linear regressions were used to establish the relationship between initial % N of leaf litter and leaf mass loss in each stream. Differences among streams were compared by ANCOVA, followed by Tukey's tests (Zar 2009).

Nitrogen immobilization (N_m) was calculated as the difference between final (N_f) and initial N concentration (N_i) of leaves and expressed as: $N_m=((N_f-N_i)/N_i)*100$. Values of nitrogen immobilization on leaves were tested against the null hypothesis that the average equalled zero (t-test). A one-way ANOVA was used to test the effect of leaf identity or stream eutrophication on N immobilization in leaves, followed by a Tukey's test (Zar, 2009).

To assess how leaf species identity influenced the assemblages of aquatic hyphomycetes (nº of spores produced by each fungal species per gram of leaf dry mass per day) and invertebrates (as nº of individuals of each species per leaf bag), data were subjected to multidimensional scaling ordination (MDS) (Clarke & Warwick, 2001). Stress values for each MDS plot indicate the goodness of representation of differences among samples (Clarke & Warwick, 2001). Data were then subjected to Unweighted Pair-Group Method Average (UPGMA) cluster

analysis, and the result was superimposed in the MDS ordination plot (Clarke & Warwick, 2001). Prior to ordination and cluster analyses, data were log (x+1) transformed and converted into a Bray Curtis similarity matrix.

MDS and cluster analyses were performed using PRIMER v6 (Software package; Plymouth Marine Laboratory, Plymouth, UK) and the remaining statistical analyses were performed with STATISTICA 8.0 for Windows (Statsoft, Tulsa, OK, USA).

4.3. Results

4.3.1. Physical and chemical characteristics of the stream water

The PCA ordination of streams according to water nutrients showed that PC1 and PC2 explained 100% of variance and defined a gradient of eutrophication, mainly represented by $N\text{-}NO_3$ and $N\text{-}NH_3$ (Fig. 4.1). This allowed us to ordinate streams according to the gradient of eutrophication as follows: Agra Stream < Oliveira Stream < Andorinhas Stream < Agrela Stream < Selho River < Couros Stream. Nutrient concentrations in the stream water ranged from 0.16-3.36 mg L^{-1} $N\text{-}NO_3$, 0.005-0.18 mg L^{-1} $N\text{-}NO_2$, 0.005-3.65 mg L^{-1} $N\text{-}NH_3$ and 0.002-0.27 mg L^{-1} $P\text{-}PO_4$. Temperature (9-15 ºC), pH (5.3-7.2) and conductivity (16-324 µS cm^{-1}) increased along the eutrophication gradient, while dissolved oxygen decreased (5.9-11.2 mg L^{-1}).

4.3.2. N immobilization in leaves

Initial N concentration in leaf litter treatments with alder (A), chestnut (C), oak (O), plane tree (P) and eucalyptus (E), alone or in mixtures, varied as follows: A (3.83%) > A+C (3.69%) > A+O (3.38%) > A+O+C (3.25%) > C (3.17%) > O (2.74%) > A+E (2.51%) > A+E+O (2.47%) > A+E+P (2.42%) = M5 (2.42%) > E (1.88%) > P (1.82%). During leaf immersion in streams, N immobilization occurred in all leaf treatments (t-test, $P<0.05$), except for oak leaves (t-test, $P=0.063$; Fig. 4.2a). N immobilization in plane tree (53% increase in N concentration) was higher than in other leaf treatments (one-way ANOVA, Tukey's test, $P<0.05$), except in eucalyptus and mixtures with all leaf species. N immobilization in leaves increased with the increase in eutrophication and was highest in the Oliveira Stream, Andorinhas Stream and Selho River (one-way ANOVA, Tukey's test, $P<0.05$; Fig. 4.2b).

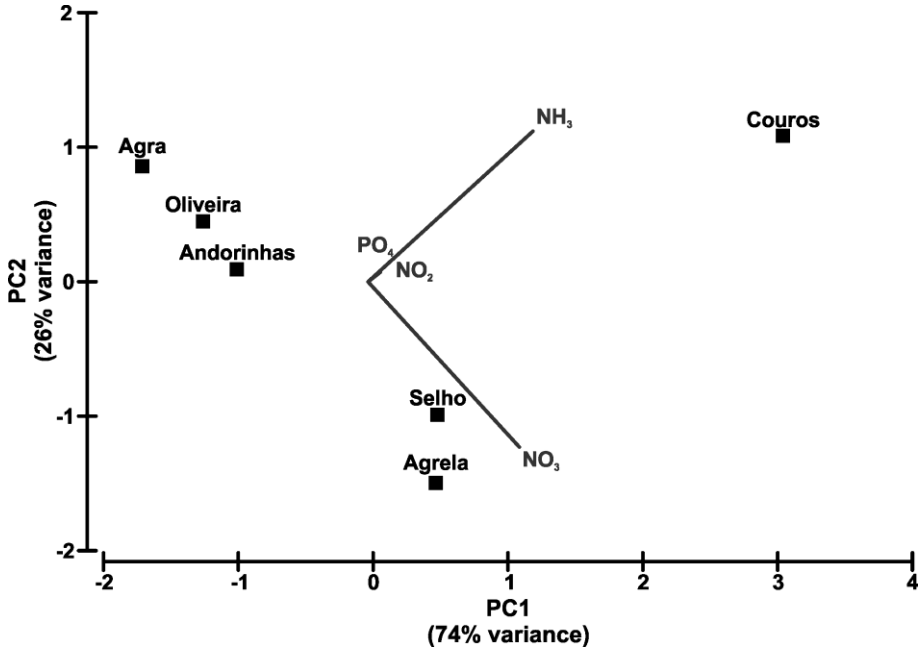

Figure 4.1 Principal Component Analysis (PCA) of the stream water variables (nutrient concentration) at six streams of the Ave River basin. The vector length reflects the contribution of that variable to these two PC axes, in relation to all possible PC axis.

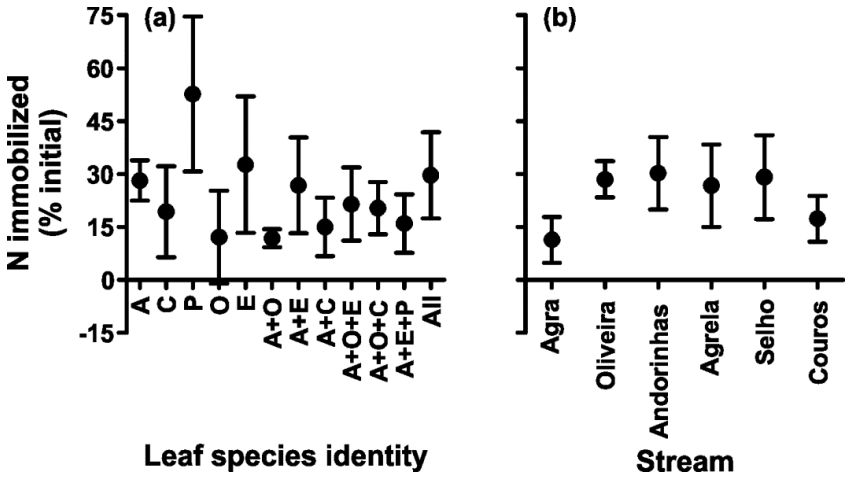

Figure 4.2 N immobilization (% of initial N) in leaves according to leaf species identity treatment (a) and stream eutrophication (b). Leaves were immersed for 38 days in six streams of the Ave River basin. Positive values indicate N immobilization. Mean ± 95%CI. Significant immobilization exists when the confidence intervals do not crosses 0. Leaf types: A, alder; C, chestnut; P, plane tree; O, oak; E, eucalyptus.

4.3.3. Biomass of fungi and invertebrates

Fungal biomass on decomposing leaves varied from 2.2 to 145 mg g^{-1} leaf dry mass. Stream, leaf species number and identity affected fungal biomass (3 way-nested ANOVA, $P<0.001$ for all factors; Table 4.1; Fig. 4.3a-c). Fungal biomass was

highest in chestnut, mixtures of alder, oak and eucalyptus, and mixtures of all leaf species (Tukey-Krammer´s test, P<0.05; Fig. 4.3a). Fungal biomass was higher in mixtures of 5 leaf species than in treatments with lower species number (Tukey-Krammer´s test, P<0.05; Fig. 4.3b). Fungal biomass was higher in the Oliveira Stream than in streams with lower or higher levels of eutrophication (Tukey-Krammer´s test, P<0.05; Fig. 4.3c). In the most eutrophic stream (Couros Stream), fungal biomass was almost 7 times lower than in the most oligotrophic stream (Agra Stream). Significant interactions were found between stream and leaf species identity, and stream and leaf species number (3 way-nested ANOVA, P<0.001 and P=0.017, respectively). Effects of leaf species identity on fungal biomass were stronger at moderate eutrophication levels (Oliveira, Andorinhas and Agrela streams, Tukey-Krammer´s test, P<0.05; data not shown).

Invertebrate biomass ranged from 1.6 to 308 mg g^{-1} leaf dry mass. Invertebrate biomass varied with the stream and leaf species identity (3 way-nested ANOVA, P<0.001 and P=0.016, respectively), but not with leaf species number (3 way-nested ANOVA, P>0.05; Table 4.1; Fig. 4.3a-c). The highest invertebrate biomass was associated with alder and chestnut leaves (Fig. 4.3a). Invertebrate biomass was lower in the Agra Stream (Tukey-Krammer´s test, P<0.05; Fig. 4.3c), tended to increase with the level of eutrophication and attained the highest value in the Selho River (Tukey-Krammer´s test, P<0.05). Invertebrate biomass in the Couros Stream was less than half the value observed in the Selho River (Tukey-Krammer´s test, P<0.05).

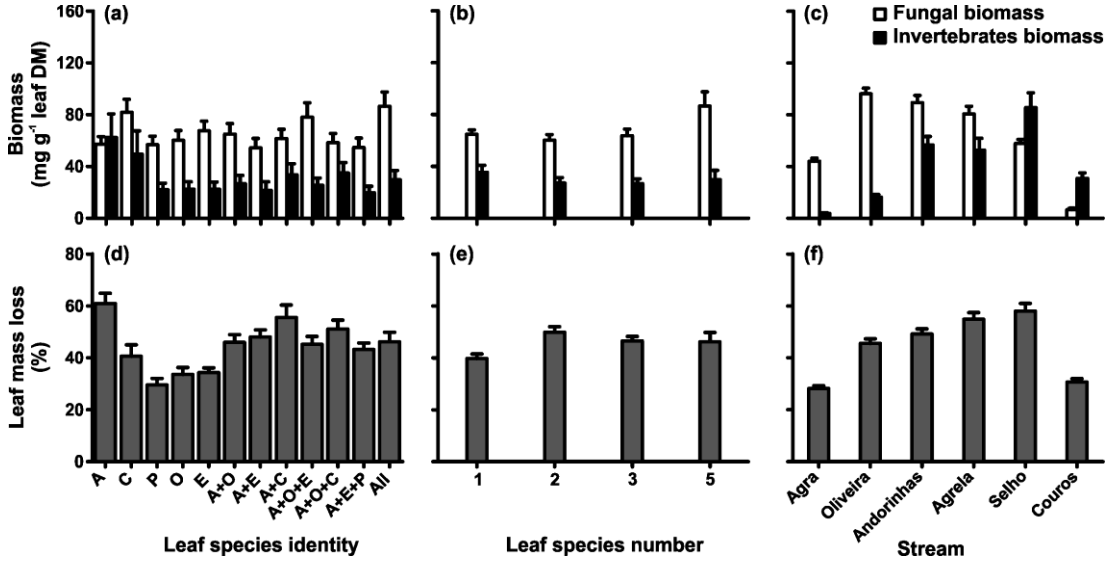

Figure 4.3 Fungal and invertebrate biomass (a-c) and leaf decomposition (d-f) after 38 days of leaf immersion in six streams of the Ave River basin with increased levels of eutrophication. Black bars, invertebrate biomass; white bars, fungal biomass. Leaf types: A, alder; C, chestnut; P, plane tree; O, oak; E, eucalyptus. M+SEM.

Table 4.1 ANOVAs of the effects of stream, species number and species identity (nested within species number) on fungal biomass, invertebrate biomass, and leaf mass loss.

	Effect	SS	DF	F	P
Fungal biomass	Stream	6090445.0	5	111.63	<0.001
	Number of species	375485.0	3	11.47	<0.001
	Identity (Number of species)	603491.0	8	6.91	<0.001
	Stream*Identity (Number of species)	1066689.0	40	2.44	<0.001
	Stream*Number of species	333221.0	15	2.04	0.017
	Error	1571380.0	144		
Invertebrate biomass	Stream	254.1	5	31.70	<0.001
	Number of Species	0.4	3	0.09	0.967
	Identity (Number of species)	31.2	8	2.43	0.016
	Stream*Identity (Number of species)	50.7	40	0.79	0.811
	Stream*Number of species	7.8	15	0.32	0.993
	Error	339.8	212		
Leaf mass loss	Stream	34948.1	5	53.84	<0.001
	Number of species	5505.2	3	14.14	<0.001
	Identity (Number of species)	17031.1	8	16.40	<0.001
	Stream*identity (Number of species)	8134.8	40	1.57	0.023
	Stream*Number of species	1805.6	15	0.93	0.535
	Error	27912.1	215		

4.3.4. Leaf decomposition

Leaf mass loss was affected by leaf species identity and number and also differed with the stream (3 way-nested ANOVA, P<0.001 for all factors; Table 4.1, Fig. 4.3d-f). Leaf mass loss was higher for alder, mixtures of alder and chestnut, and mixtures of alder, chestnut and oak (Tukey-Krammer´s test, P<0.05; Fig. 4.3d). Leaf mass loss was higher in mixtures of 2 and 3 species compared to single species treatments (Tukey-Krammer´s test, P<0.05), but no differences between leaf mixtures were found (Tukey-Krammer´s test, P>0.05; Fig. 4.3e). Leaf mass loss was low at the most oligotrophic site (Agra Stream), increased with eutrophication until a certain level (Selho River) and then decreased at the most eutrophic site (Couros Stream) (Tukey-Krammer´s test, P<0.05; Fig. 4.3f). A significant interaction was found between effects of leaf identity and stream on leaf mass loss (3 way-nested ANOVA, P=0.023; Table 4.1). Apart from the Couros Stream, positive linear relationships between initial % of N in leaves and leaf mass loss were found (linear regression, P<0.05, Fig. 4.4; Table 4.2). Moreover, the dependence of leaf mass loss on % of N in leaves increased with eutrophication as shown by the differences in the slopes of these relationships (ANCOVA, P<0.05).

Leaf diversity effects, assessed as deviation from additivity (difference between the observed leaf mass loss in mixtures and the expected values based on the weighed sum of individual mass losses), increased with increasing number of leaf species in mixtures. Significant positive diversity effects were found in mixtures of 3 and 5 species (t-tests, $P<0.05$; Fig. 4.5a). Effects of leaf diversity were positive in the less eutrophic streams, namely Agra and Oliveira streams (t-tests, $P<0.05$; Fig. 4.5b), but not in streams with higher levels of eutrophication (t-tests, $P>0.05$).

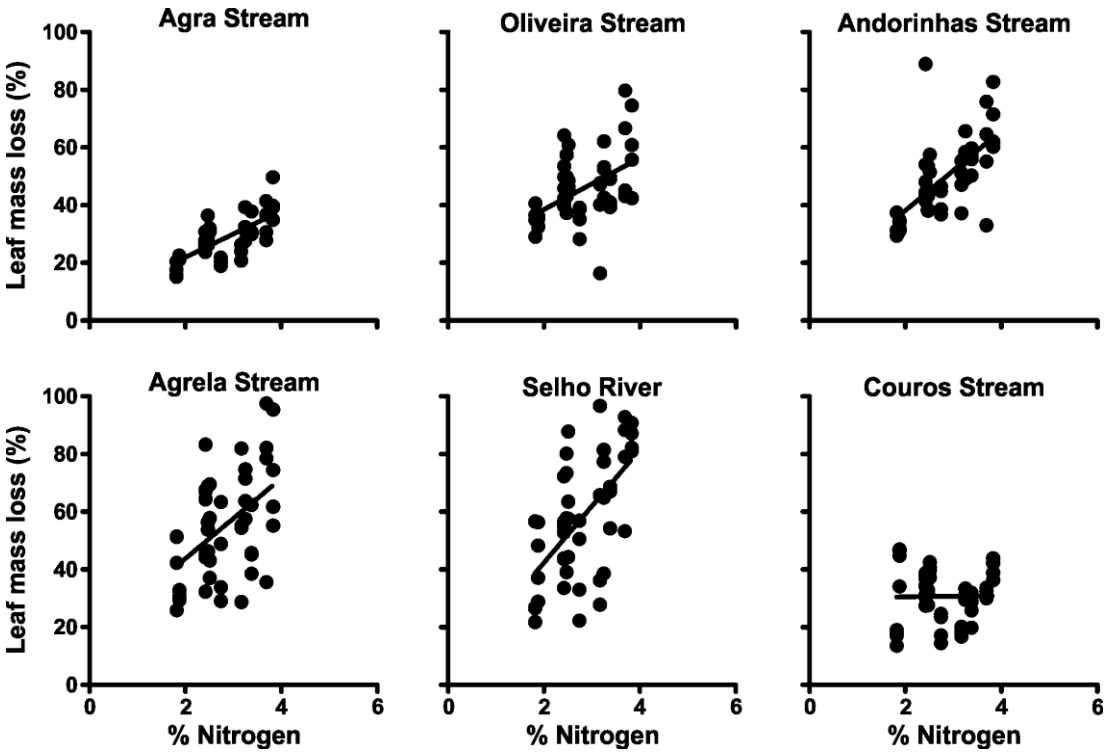

Figure 4.4 Relationship between initial N concentration in leaves (%) and leaf decomposition after 38 days of leaf immersion in six streams of the Ave River basin. Data was fitted to linear regression.

Table 4.2 Linear regressions of the relationship between initial N concentration in leaves and leaf mass loss after 38 days of leaf immersion in six streams of the Ave River basin, as shown in Fig. 4.4. Slopes were compared by ANCOVA followed by Tukey's tests.

Stream	Equation	P	r^2	Tukey's test
Agra	Y=8.07X+5.74	< 0.0001	0.51	a
Oliveira	Y=8.92X+20.65	0.0006	0.23	b
Andorinhas	Y=13.84X+10.42	< 0.0001	0.42	b, c
Agrela	Y=13.91X+15.90	0.0004	0.24	c, d
Selho	Y=19.59X+3.13	< 0.0001	0.36	d
Couros	Y=0.20X+30.11	0.9226	<0.01	a

Similar letters indicate no significant differences between slopes.

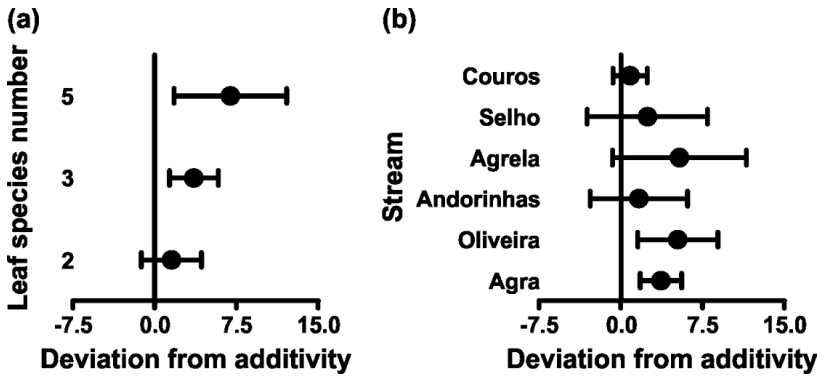

Figure 4.5 Deviation from additivity (observed minus expected litter mass loss) at each level of leaf species number (a) and at each level of stream eutrophication (b). Negative deviation from additivity indicates antagonistic response (lower leaf litter decomposition), and positive deviation indicates synergistic response (higher leaf litter decomposition) of litter mixtures. Mean ± 95% CI. Significant litter mixtures effects exist when the confidence intervals do not cross 0.

4.3.5. Fungal and invertebrate assemblages

The MDS ordination of aquatic hyphomycete species sporulating on leaves separated assemblages in leaf mixtures from those in single leaf species, especially those in eucalyptus leaves (Fig. 4.6). Within leaf mixtures, fungal assemblages in mixtures containing eucalyptus leaves shared 85% of similarity and were separated from fungal assemblages in other leaf mixtures.

The MDS ordination of leaf-associated invertebrate assemblages separated assemblages in single leaf species from those in leaf mixtures, and assemblages within each of these groups shared 80% similarity (Fig. 4.6). Within single leaf species, invertebrate assemblages associated with alder and chestnut leaves grouped together and shared 85% of similarity.

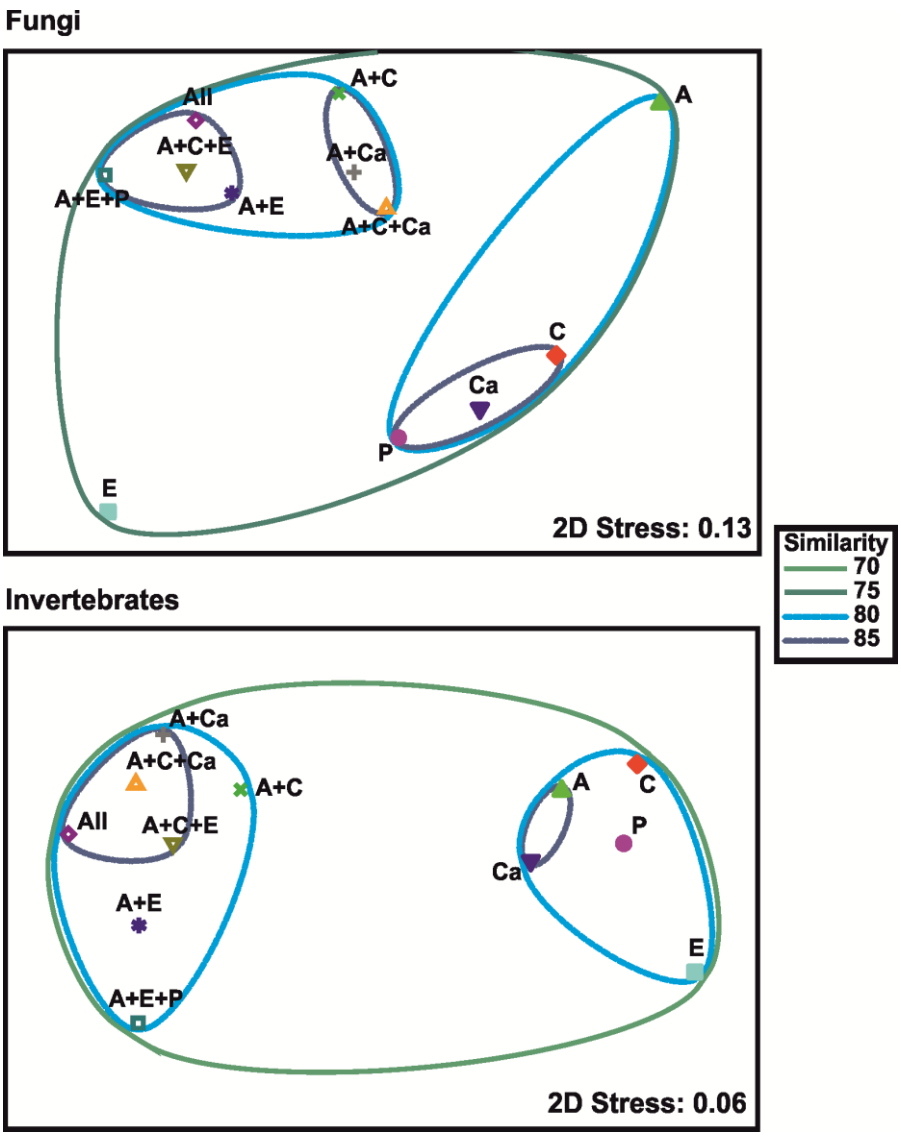

Figure 4.6 Two-dimensional plots of Multidimensional Scaling ordination (Bray-Curtis similarity) of taxa of fungi (sporulation rate) and invertebrates (abundance) on leaves, according to leaf species identity. Stress values indicate that values are not randomly distributed. Circles indicate similarities within assemblages superimposed in the MDS from cluster analysis.

4.4. Discussion

Our results suggested that riparian plant diversity might have positive effects on litter decomposition mediated by fungi and invertebrates in streams. This agrees with other studies indicating that effects of leaf litter diversity on litter decomposition are mostly positive (Gartner & Cardon, 2004; Lecerf *et al.*, 2011). In addition, we found that plant diversity effects increased with the increase in leaf species number (Fig. 4.5a), suggesting that the loss of riparian tree species might affect stream ecosystem functioning by weakening interactions between leaves and decomposers in leaf species mixtures. Moreover, our results showed that eutrophication could

modulate diversity effects: synergistic diversity effects on leaf decomposition were observed in streams with lower nutrient levels (Agra and Oliveira Streams) but not in eutrophic streams (Fig. 4.5b). To our knowledge, the only study carried out to assess the combined effects of nutrient enrichment and litter diversity on litter decomposition in streams found antagonistic effects of litter species mixtures on decomposition, but these effects were lost when nutrient concentrations in the stream water increased (Rosemond *et al.*, 2010). Nevertheless, the level of litter diversity used in that study (up to 3 species) was lower than that of our study (up to 5 species) and the addition of nutrients to the stream water resulted in much lower nutrient concentrations (up to 0.39 mg L^{-1} DIN and 0.06 mg L^{-1} SRP) than those found in our eutrophic streams (up to 3.36 mg L^{-1} $N\text{-}NO_3$, 3.65 mg L^{-1} $N\text{-}NH_3$, 0.27 mg L^{-1} $P\text{-}PO_4$ and 0.18 mg L^{-1} $N\text{-}NO_2$).

Plant litter decomposition is expected to be faster at higher levels of litter diversity because a wider range of resources is available to litter decomposers. However, leaf litter might not be the sole source of nutrients for organisms involved in leaf decomposition. Aquatic fungi obtain nutrients mainly from leaf litter, but they also have the ability of using nitrogen and phosphorus directly from the stream water (Suberkropp & Chauvet, 1995). So, in streams with higher nutrient concentrations, microbes may take benefit of the available nutrients in the stream water, contributing to explain the absence of positive effects of leaf mixtures found in our eutrophic streams. Also in these streams, N immobilization, especially in lower quality leaf types such as the plane tree, may have led to a homogenization of resource quality and to a decrease of available niches, increasing competition between invertebrates and weakening the effects of leaf litter diversity (Bastian *et al.*, 2008).

The effects of litter diversity are frequently explained by the identity of species that constitute the mixture rather than the number of litter species (Swan & Palmer, 2004, 2006; Lecerf *et al.*, 2007). The effects of leaf identity on leaf decomposition may result from differences in litter quality, such as concentration of lignin, cellulose, nitrogen or phosphorus (Lecerf & Chauvet, 2008; Schindler & Gessner, 2009; Fernandes *et al.*, 2012). In our study, treatments containing leaves with higher N content, namely alder alone or in mixtures with chestnut, showed the highest leaf decomposition. Indeed, apart from the Couros Stream, a positive linear relationship between leaf mass loss and initial N concentration in leaves was observed, and this relationship became stronger as eutrophication increased (Fig. 4.4). The strengthening of relationships between litter quality and decomposition in eutrophic streams might have occurred because fungi benefit from inorganic nutrients in the stream water leading to an enhanced use of leaf carbon. Moreover, the enhanced

microbial nutrient uptake from the water column (Cross *et al.*, 2003; Gulis & Suberkropp, 2003; Ferreiro *et al.*, 2011) and the increased fungal activity (Sridhar & Bärlocher, 2000; Gulis & Suberkropp, 2003; Ferreira & Chauvet, 2011) in nutrient enriched streams may also contribute to improve litter quality to invertebrate shredders leading to an overall increase in litter decomposition. The lack of leaf diversity effects in the most eutrophic stream (Couros Stream) might be due to the inhibition of fungal and invertebrate activity on leaves, as supported by the very low fungal biomass and the absence of shredders at this site. Indeed, in the Couros Stream, invertebrates were mainly Chironomidae (not shown), which are considerably generalists covering a wide trophic spectrum, from filtration to predation (Oscoz *et al.*, 2011); hence, their contribution to leaf decomposition could be less dependent on litter quality.

In our study, effects of leaf litter diversity and/or identity on leaf decomposition were accompanied by shifts in fungal and invertebrate assemblages. Fungal assemblages on single species separated from those on leaf mixtures, supporting that changes in litter diversity would condition the structure of fungal communities. In addition, fungal assemblages in mixtures with eucalyptus leaves further separated from the others. Eucalyptus leaves contain oils and tannic acids that inhibit the growth of aquatic fungi (Canhoto & Graça, 1999) and, thus, we might expect that some fungi would avoid eucalyptus leaves or mixtures containing it. Decreased diversity of aquatic fungi and shifts in community composition were previously found in streams crossing monocultures of eucalyptus compared with streams bordered by native mixed forests (Bärlocher & Graça, 2002). In our study, invertebrate assemblages in leaf mixtures also differed from those in single leaf species. Within single species, invertebrate assemblages associated with leaves with higher initial N concentration (alder and chestnut) separated from the others. These findings suggest that alterations in riparian plant diversity may change the structure of aquatic communities in detritus food webs in streams.

A closer look on the selected streams showed that sites could be ordinated according to a gradient of eutrophication defined mainly by nitrates and ammonia. Streams with moderate and moderately high levels of eutrophication had higher leaf decomposition than oligotrophic (Agra Stream) or hypertrophic streams (Couros Stream). These results are consistent with a recent survey in 100 streams in Europe which showed an hump-shaped relationship between leaf decomposition and the eutrophication level (Woodward *et al.*, 2012). In addition, the biomass of fungi and invertebrates associated with decomposing leaves followed the same pattern:

overall biomass increased with the eutrophication level but decreased at the most eutrophic stream (Couros Stream).

The role of invertebrates and microbes in leaf decomposition may change in eutrophic streams (Pascoal *et al.*, 2005). In our study, fungal biomass was higher than that of invertebrates in the majority of the streams excepting the two most eutrophic ones (Selho River and Couros Stream). At the Couros Stream, oxygen concentration in the stream water was low (5.9 mg L^{-1}). Hypoxic and anoxic conditions that are usually associated with eutrophic and hypertrophic environments may lead to suppression of microbial activity (Pascoal & Cássio, 2004). In addition, invertebrate assemblages in the Selho River and the Couros Stream were mainly constituted by Oligochaeta and Chironomidae, respectively (not shown), while shredders, the major leaf-eating invertebrates in streams (Allan & Castillo, 2007), were almost absent (not shown). The presence of Oligochaeta and Chironomidae in high quantities might have prompted leaf decomposition (Pascoal *et al.*, 2003; Pascoal *et al.*, 2005) not directly by feeding on leaves but rather by promoting physical fragmentation due to their mobility and feeding on FPOM in the leaf surface (Chauvet *et al.*, 1993).

Overall results suggest that effects of riparian plant diversity on stream ecosystem functioning are modulated by eutrophication. Both the quality and number of plant species affected leaf decomposition by microbes and invertebrates in streams. Specifically, we found synergistic effects of litter diversity on leaf decomposition. However, positive effects of leaf species number on leaf decomposition were only observed in the less eutrophic streams. This suggests that oligotrophic streams are more dependent on the number of riparian plant species than eutrophic streams. If so, riparian plant diversity should be preserved in oligotrophic systems to maintain leaf decomposition. On the other hand, the positive effects of litter quality (as % of leaf N) on leaf decomposition were strengthened by increased nutrient concentrations in the stream water, suggesting that litter decomposition depends more on the quality than the number of riparian plants in eutrophic streams. These findings lend support to the hypothesis that eutrophication modulates leaf diversity effects on leaf decomposition with potential implications for stream ecosystem functioning. The control and recovery of eutrophied streams is within the goals of the Water Framework Directive (Water Framework Directive, 2000/60/EC) which intends to achieve a “good status” for all of Europe's surface waters and groundwater by 2015. If we believe that those goals are achievable and water quality of eutrophic streams will be improved, we might observe an intensification of leaf diversity effects on litter decomposition in streams in the near future.

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Chapter 5

Increased temperature may augment the positive effects of nutrients on plant litter decomposition in streams

Abstract

Climate change scenarios point to an increase in global temperature and to alterations in precipitation regimes, which may increase nutrient concentration in water bodies. In forested streams, decomposition of allochthonous organic matter is a key ecosystem process that is affected by the quality of plant litter entering the streams, water temperature and nutrient concentrations in the stream water. Although the individual effects of all these factors on plant litter decomposition in streams have been investigated, their combined effects on this pivotal ecosystem process remains poorly explored. We examined the interactive effects of increased temperature, concentration of inorganic nutrients and litter quality on leaf decomposition and activity of the associated aquatic microbes. Leaves of alder and oak were immersed for 10 d in a stream (NW Portugal) to allow microbial colonization, and then were exposed in microcosms to $N\text{-}NO_3$ (0.09-5 mg L^{-1}; 6 levels) and $P\text{-}PO_4$ (0.003-0.3 mg L^{-1}; 3 levels), alone or in all possible combinations. One set of microcosms was kept at 12 ºC, a temperature typically found in Iberian streams in autumn, and the other set at 18 ºC to simulate a warming scenario. Nitrogen immobilization in alder leaves was higher than in oak leaves and increased with N concentration and temperature in the stream water for both leaf types. Overall, microbial activity increased asymptotically (Michaelis-Menten kinetic) with N concentration in the stream water. Increased temperature led to an increase in maximum fungal activity and to a decrease in N concentration needed to achieve maximum fungal activity, especially on alder leaves. This suggests that, under the predicted warming scenario, maximum fungal activity may be attained in streams with lower nutrient levels, especially those receiving high quality leaf litter inputs, which may result in faster leaf decomposition, leading to changes in detritus food webs.

5.1. Introduction

Climate change is arguably an emerging global threat which may have profound impacts on physical and biological systems mainly due to changes in temperature and precipitation regimes (Rosenzweig *et al.*, 2007). It is predicted that heavy precipitation may increase surface runoff and erosion escalating nutrient loading in streams (Jeppesen *et al.*, 2011). In addition, alternate drought periods will decrease summer flow and increase evapotranspiration leading to less dilution of nutrient inputs (Murdoch *et al.*, 2000; Whitehead *et al.*, 2009). Another subtle impact of the ongoing climate change is an increase in global temperature. It is expected that by the end of 21st century, Europe would witness an increase in air temperature until 6.4 °C (Meehl *et al.*, 2007). Climate change is also expected to shift plant species dominance patterns (Bakkenes *et al.*, 2002; Kardol *et al.*, 2010), which may alter the quality of plant litter.

In low-order forested streams, allochtonous plant litter constitutes the main source of food and energy for aquatic biota (Vannote *et al.*, 1980). Aquatic fungi are the dominant microbial decomposers of leaf litter in streams and play a key role in transferring carbon and energy from plant litter to invertebrate detritivores (Gessner *et al.*, 2007). Freshwater ecosystems are markedly vulnerable to climate change as they are comparatively isolated, patchy and greatly exploited by humans (Woodward *et al.*, 2010). A number of studies has assessed the effects of plant litter quality (Gulis *et al.*, 2004; Ardón *et al.*, 2006; Ferreira *et al.*, 2006; Gulis *et al.*, 2006) and increased nutrient concentration in the stream water (Robinson & Gessner, 2000; Grattan & Suberkropp, 2001; Menéndez *et al.*, 2011) on plant litter decomposition in streams. Increases in inorganic nutrients in streams were found to stimulate microbial activity and plant-litter decomposition until a certain point, above which it came to a standstill (Rosemond *et al.*, 2002; Ferreira *et al.*, 2006; Gulis *et al.*, 2006). However, very high nutrient concentrations may become toxic to decomposers inhibiting leaf decomposition (Woodward *et al.*, 2012). Higher quality leaf litter is known to decompose faster (Webster & Benfield, 1986; Sampaio *et al.*, 2001; Lecerf & Chauvet, 2008) probably as a result of high N or P content in leaves (Chadwick & Huryn, 2003; Lecerf & Chauvet, 2008). However, till date, there is no consensus regarding the interactive effects of litter quality and stream water nutrients on leaf decomposition. Sometimes, increased nutrient concentrations in the stream water have stronger effects on low quality leaves (Gulis *et al.*, 2006) while other times effects were reported to be stronger on high quality leaves (Ardón *et al.*, 2006). Because aquatic fungi on decomposing leaves can obtain nutrients from both

stream water and plant litter (Suberkropp, 1998), it is important to discriminate the relative effects of nutrients from plant litter and from stream water on litter decomposition mediated by fungi.

Leaf decomposition can increase with temperature (Fernandes *et al.*, 2009; Friberg *et al.*, 2009; Ferreira & Chauvet, 2011b; Geraldes *et al.*, 2012), probably as a result of increased metabolic activity of aquatic fungi (Rajashekhar & Kaveriappa, 2000; Ferreira & Chauvet, 2011b). Moreover, temperature effects on litter decomposition are likely to interact with other variables such as leaf litter quality or inorganic nutrients in the stream water. Results from manipulative experiments suggest that climate warming will exacerbate the stimulatory effects of nutrient enrichment on fungal activity and leaf decomposition (Ferreira & Chauvet, 2011b) and will strongly control litter decomposition, and fungal activities and community structure, if only slight modifications in litter quality occur (Ferreira & Chauvet, 2011a). Although changes in plant litter quality and increases in nutrients and temperature in the stream water are the anticipated consequences of global change (Peñuelas & Matamala, 1990; Alcamo *et al.*, 2007; Meehl *et al.*, 2007), their interactive effects on litter decomposition in streams are still poorly understood and demands further attention.

This study was designed to examine the effects of increased stream water temperature, and litter quality (higher and lower initial N content) on leaf litter decomposition driven by microbes along a gradient of inorganic nutrients (6 levels of N; 3 levels of P). We hypothesized that an increase in water temperature and nutrient supply from both stream water and leaves would augment fungal activity and leaf decomposition (Lecerf & Chauvet, 2008; Ferreira & Chauvet, 2011b). If so, we expected that maximum fungal activity would be attained at lower levels of inorganic nutrients in the stream water in the higher quality leaf type at higher temperature. We used microcosms with naturally occurring microbial assemblages in streams, and effects were assessed on leaf decomposition, fungal biomass and reproduction, and leaf nitrogen immobilization.

5.2. Methods

5.2.1. Sampling site and microbial colonization

The stream site is located at the National Park of Peneda-Gerês, Northwest of Portugal (longitude 41°46’07.64’’N, latitude 8°08’49.07’’W and elevation 857 m). Conductivity, pH and dissolved oxygen concentration in the stream water was

measured *in situ* (Multiline F/set 3 no. 400327, WTW, Weilheim, Germany) and water samples were collected for quantification of nitrate (HACH kit, program 355), nitrite (HACH kit, program 371), ammonia (HACH kit, program385) and phosphate (HACH kit, program 480). Data from physical and chemical analysis of the stream water indicated slightly acidic pH (5.5), low conductivity (15 µS cm^{-1}), high dissolved oxygen concentration (10 mg L^{-1}), and low concentration of inorganic nutrients (0.09 mg L^{-1} $N\text{-}NO_3$, 0.003 mg L^{-1} $P\text{-}PO_4$, 0.008 mg L^{-1} $N\text{-}NO_2$ and 0.04 mg L^{-1} $N\text{-}NH_3$).
Air dried leaves of alder (*Alnus glutinosa* (L.) Gaertn.) and oak (*Quercus robur* L.), collected in autumn 2008 from single trees, were leached for 1 h in deionised water and cut into 12 mm disks. Sets of 40 disks of each leaf type were placed into fine-mesh bags (16×20 cm, 0.5-mm mesh size to minimize the entrance of invertebrates), in a total of 216 bags. On 10th December 2008, leaf bags were immersed at the study site to allow microbial colonization.

5.2.2. Microcosm assay

After 10 days of leaf immersion, bags were retrieved from the stream and transported to the laboratory. Leaf disks from each bag were rinsed with deionised water and placed into 150 mL Erlenmeyer flasks with 70 mL of sterilized stream water (120 °C, 20 min). The microcosms were supplemented with KNO_3 (Panreac, Barcelona, Spain) at final $N\text{-}NO_3$ concentrations of 0.3, 0.6, 1.2, 2 and 5 mg L^{-1}, and with KH_2PO_4 (Riedel-deHaën, Seelze, Germany) at final $P\text{-}PO_4$ concentrations of 0.03 and 0.3 mg L^{-1}, alone or in all possible combinations (3 replicates). One set of microcosms was exposed to 12 ºC, a temperature commonly found in streams of this region in autumn (Pascoal *et al.*, 2003; Duarte *et al.*, 2009), and the other set was exposed to 18 ºC to simulate a warming scenario. Microcosms were incubated under shaking at 120 rpm, for 18 days, and solutions were renewed every 6 days.
At the end of the experiment, leaf disks were used to quantify leaf mass loss, fungal biomass, and nitrogen concentration in leaf litter. Water solutions containing conidial suspensions were mixed with formalin (2% final concentration) and stored until used.

5.2.3. Leaf decomposition

The remaining leaf disks from each microcosm were freeze-dried (Christ alpha 2–4, B. Braun, Germany) to constant mass (± 48 h) and weighed to the nearest 0.01 mg. The initial dry mass of alder and oak leaves was determined using the content of

three leaf bags of each leaf type immersed for 5 min in the stream at the beginning of the study.

5.2.4. Fungal biomass and reproduction

A set of 6 freeze-dried leaf disks from each microcosm was used to determine ergosterol concentration as a measure of fungal biomass on leaves. Lipids were extracted from leaf disks by heating (80 ºC for 30 min) in 8 g L^{-1} of KOH/methanol, purified by solid-phase extraction and quantified by high performance liquid chromatography (Gessner, 2005).

Fungal reproduction was estimated based on identification and counting of released conidia. Conidial suspensions were mixed with 200 µL of 0.5% Tween 80, filtered (0.45-µm pore size, Millipore, Billerica, MA, U.S.A.), and the retained conidia were stained with 0.05% cotton blue in lactic acid. At least 300 conidia were identified and counted under a light microscope at 400x magnification (Leica Biomed, Heerbrug, Switzerland).

5.2.5. Nitrogen immobilization in leaf litter

Equal numbers of leaf disks from each replicate treatment were pooled and ca. 2 mg of the ground leaf material was used for nitrogen quantification using an elemental analyzer (CHNS-O EA-1108, Carlo Erba Instruments, ThermoScientific, Massachusetts, USA) in the Centro de Apoio Científico e Tecnolóxico á Investigación (CACTI, University of Vigo, Spain). Initial N concentration was 3.26% in alder leaves and 2.32% in oak leaves. Nitrogen immobilization (N_m) was calculated as the difference between final (N_f) and initial N concentration (N_i) of leaves and expressed as percentage of initial N concentration (N_i) in leaves: $N_m=((N_f-N_i)/N_i)*100$. Positive values represent N immobilization.

5.2.6. Statistical analyses

A two-way ANOVA was used to test the effects of N and P concentrations in the stream water on leaf decomposition, fungal biomass, fungal reproduction and N immobilization. Effects were tested individually for each leaf type. Because N had highly significant effects and P had no effects on all tested parameters (2-way ANOVA, $P<0.02$ and $P>0.05$, respectively), we further described the effects of N on the measured endpoints. A Michaelis-Menten model was used to establish the relationships between N concentration in the stream water and leaf decomposition, fungal biomass, fungal reproduction or N immobilization as $V=(V_{max}*[N])/(K_m+[N],$

where V_{max} is the maximum function, K_m is the N concentration at which half of maximum function is achieved, and [N] is N concentration in the stream water. Whenever data were poorly fitted to a Michaelis-Menten model, a Lorentzian model or a linear model was applied. The Lorentzian model is derived from the Gaussian model and is described as Y=*Amplitude*/(1+(([N]-*Center*)/*Width*)^2, where *Amplitude* is the maximum function, *Center* is the N concentration at which *Amplitude* is achieved, *Width* is a measure of the distribution width in the same units as [N]. The linear regression is described as Y=*slope**[N]+Y_0, where the *slope* is the variation in function per unit of [N] in the stream water, and Y_0 is the Y intercept. Differences in the model parameters between temperatures and leaf types were assessed using extra sum-of-squares F test.

ANOVAs were carried out in STATISTICA 6.0 for Windows (Statsoft, Tulsa, OK, USA). Regression analyses and model parameter comparisons were done in Prism 4.0 for Windows (GraphPad software Inc., San Diego, CA).

5.3. Results

5.3.1. Fungal reproduction

Sporulation rates of fungi associated with alder leaves increased with N concentration in the stream water until moderate levels and then decreased at both 12 ºC and 18 ºC (Lorentzian model; Fig. 5.1a, Table 5.1). Maximum fungal sporulation rates estimated by the model (*Amplitude*) were 13x10^5 and 9.5x10^5 spores g^{-1} leaf dry mass d^{-1} at 12 ºC and 18 ºC, respectively, and differed significantly between temperatures ($P<0.05$; Table 5.2). Maximum fungal sporulation rate on alder leaves was predicted to be attained at N concentration (*Center*) almost three-times higher at 12 ºC (3.05 mg L^{-1} N-NO_3) than at 18 ºC (1.07 mg L^{-1} N-NO_3) ($P<0.05$; Table 5.2).

At 12 ºC, fungal sporulation rates on oak leaves increased linearly with the concentration of N in the stream water until the highest tested concentration (5 mg L^{-1} N-NO_3), in which sporulation rate was 1.39x10^5 spores g^{-1} leaf dry mass d^{-1} (Fig. 5.1b, Table 5.1). At 18 ºC, fungal sporulation rates on oak leaves increased asymptotically with N concentration in the stream water (Michaelis-Menten model; Fig. 5.1b, Table 5.1) and attained a maximum of 1.3x10^5 spores g^{-1} leaf dry mass d^{-1} (V_{max}). The concentration of N in the stream water needed to achieve half of maximum fungal sporulation (K_m) in oak leaves was 0.20 mg L^{-1} N-NO_3 (Table 5.1).

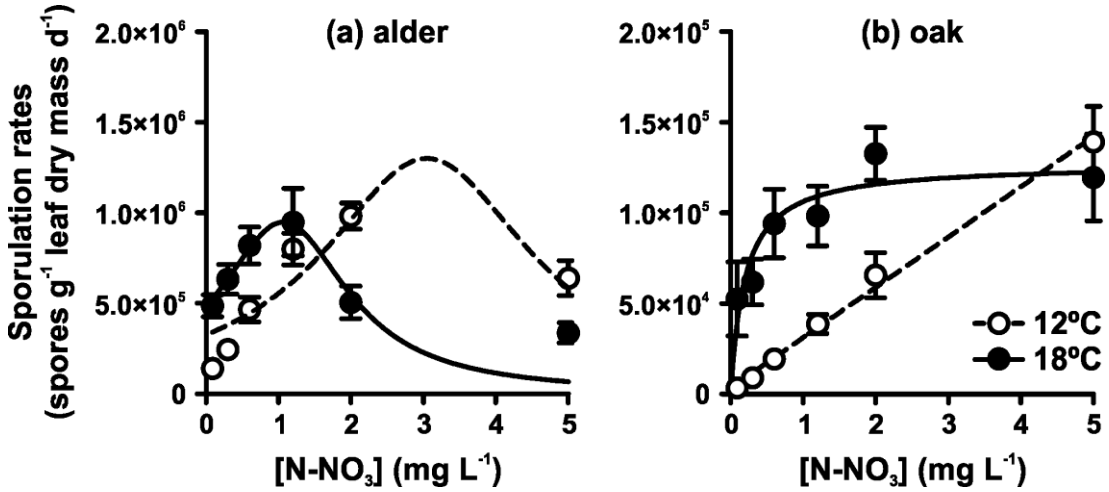

Figure 5.1 Sporulation rates of aquatic fungi associated with alder (a) and oak (b) leaves exposed to increasing N concentrations in the stream water during 18 days in microcosms at 12 ºC and 18 ºC. Data from alder leaves were fitted to a Lorentzian model. Data from oak at 12 ºC were fitted to a linear regression, while at 18 ºC were fitted to a Michaelis-Menten model. M ± SEM; n = 9.

Table 5.1 Model parameters of the relationship between nitrate concentration in the stream water (N) and fungal reproduction, fungal biomass, N immobilization or leaf decomposition. Alder leaves (A) and oak leaves (O) were colonized in a stream and then exposed for 18 days in microcosms to 12 ºC or 18 ºC. Lorentzian model: $Y=Amplitude/(1+(([N]-Center)/Width)^2)$; Michaelis-Menten model: $V=(V_{max}*[N])/(K_m+[N])$; Linear model: $Y=slope*[N]+Y_0$. Model parameters are defined in Material and Methods.

	Leaf Type	Temperature	Model	Parameter	Parameter value	r^2
Fungal reproduction	A	12 ºC	Lorentzian	*Amplitude* (spores g^{-1} leaf dry mass d^{-1})	$13x10^5$	0.57
				Center (mg L^{-1} $N\text{-}NO_3$)	3.05	
				Width (mg L^{-1} $N\text{-}NO_3$)	1.77	
		18 ºC	Lorentzian	*Amplitude* (spores g^{-1} leaf dry mass d^{-1})	$9.5x10^5$	0.23
				Center (mg L^{-1} $N\text{-}NO_3$)	1.07	
				Width (mg L^{-1} $N\text{-}NO_3$)	1.09	
	O	12 ºC	Linear regression	*slope* (spores g^{-1} leaf dry mass d^{-1} mg^{-1} L^{-1} $N\text{-}NO_3$)	$0.28x10^5$	0.73
				Y_0 (spores g^{-1} leaf dry mass d^{-1})	$0.03x10^5$	
		18 ºC	Michaelis-Menten	V_{max} (spores g^{-1} leaf dry mass d^{-1})	$1.3x10^5$	0.19
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.20	

Table 5.1 (Continued)

	Leaf Type	Temperature	Model	Parameter	Parameter value	r^2
Fungal biomass	A	12 ºC	Michaelis-Menten	V_{max} (µg ergosterol g^{-1} leaf dry mass)	376.7	0.30
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.06	
		18 ºC	Michaelis-Menten	V_{max} (µg ergosterol g^{-1} leaf dry mass)	485.6	0.10
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.02	
	O	12 ºC	Michaelis-Menten	V_{max} (µg ergosterol g^{-1} leaf dry mass)	423.9	0.64
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.22	
		18 ºC	Michaelis-Menten	V_{max} (µg ergosterol g^{-1} leaf dry mass)	669.0	0.51
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.09	
N Immobilization	A	12 ºC	Michaelis-Menten	V_{max} (% initial)	61.65	0.46
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.04	
		18 ºC	Michaelis-Menten	V_{max} (% initial)	69.04	0.27
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.02	
	O	12 ºC	Michaelis-Menten	V_{max} (% initial)	42.53	0.33
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.04	
		18 ºC	Michaelis-Menten	V_{max} (% initial)	49.11	0.29
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.03	
Leaf decomposition	A	12 ºC	Michaelis-Menten	V_{max} (% leaf mass loss)	66.13	0.47
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.08	
		18 ºC	Michaelis-Menten	V_{max} (% leaf mass loss)	67.14	0.45
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.02	
	O	12 ºC	Michaelis-Menten	V_{max} (% leaf mass loss)	29.32	0.21
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.02	
		18 ºC	Michaelis-Menten	V_{max} (% leaf mass loss)	34.96	0.13
				K_m (mg L^{-1} $N\text{-}NO_3$)	0.01	

5.3.2. Fungal biomass

Fungal biomass increased asymptotically with N concentration in the stream water for both leaf types and temperatures (Michaelis-Menten model; Fig. 5.2). According to the models, maximum fungal biomass (V_{max}) was significantly higher on oak leaves (423.9 and 669.0 µg ergosterol g^{-1} leaf dry mass at 12 ºC and 18 ºC, respectively) than on alder leaves (376.7 and 485.6 µg ergosterol g^{-1} leaf dry mass at 12 ºC and 18 ºC, respectively) at 18 ºC ($P<0.05$; Table 5.1, Table 5.2). Moreover, maximum fungal biomass (V_{max}) was higher at 18 ºC than at 12 ºC in both leaf types ($P<0.05$; Table 5.2). Nitrogen concentration in the stream water needed to achieve half of maximum fungal biomass (K_m) was lower at 18 ºC than at 12 ºC for both leaf types ($P<0.05$; Table 5.2). K_m values for fungal biomass were lower on alder than on oak leaves at both temperatures ($P<0.05$; Table 5.2).

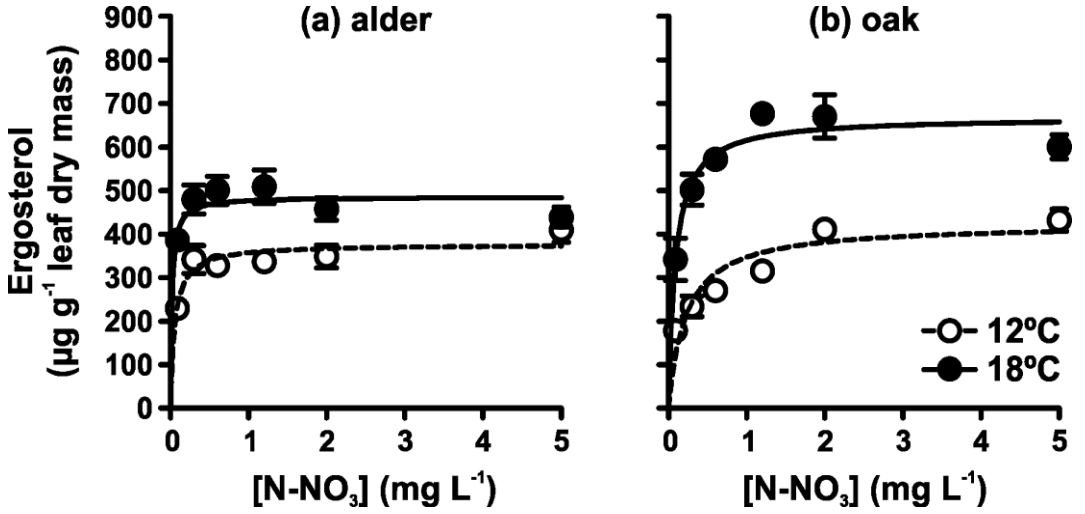

Figure 5.2 Fungal biomass, as ergosterol concentration, associated with alder (a) and oak (b) leaves exposed to increasing N concentrations in the stream water during 18 days in microcosms at 12 ºC and 18 ºC. Data were fitted to a Michaelis-Menten model. M ± SEM; n = 9.

5.3.3. Nitrogen immobilization in leaf litter

Nitrogen immobilization in both leaf types increased asymptotically with N concentration in the stream water (Michaelis-Menten model, Fig. 5.3, Table 5.1). Maximum N immobilization (V_{max}) was higher in alder (62% and 69% at 12 ºC and 18 ºC, respectively) than in oak (43% and 49% at 12 ºC and 18 ºC, respectively) ($P<0.05$; Table 5.1, Table 5.2). For both leaf types, V_{max} was higher at 18 ºC than at 12ºC ($P<0.05$; Table 5.2). The nitrogen concentration needed to achieve half of maximum N immobilization (K_m) was similar in both leaf types and temperatures ($P>0.05$; Table 5.2).

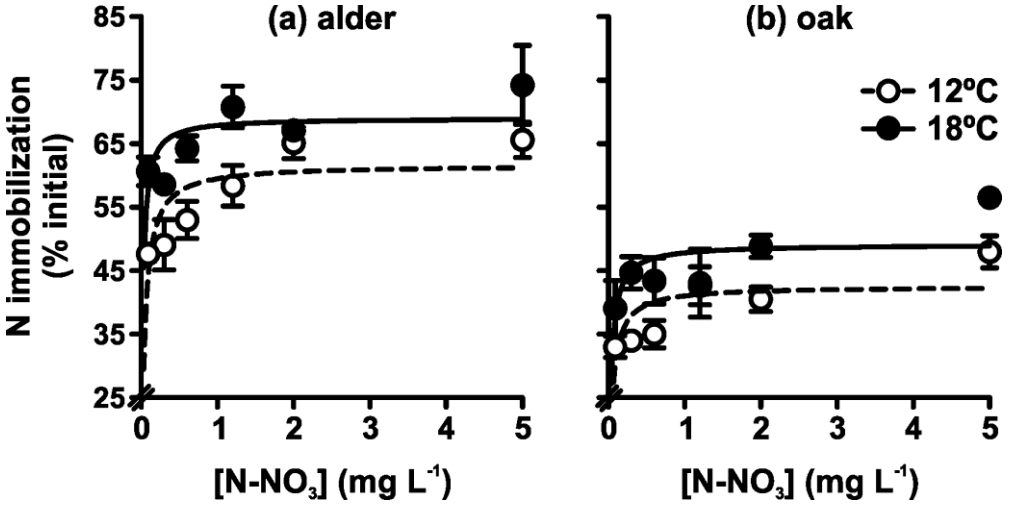

Figure 5.3 Nitrogen immobilization on alder (a) and oak (b) leaves exposed to increasing N concentrations in the stream water during 18 days in microcosms at 12 ºC and 18 ºC. Data were fitted to a Michaelis-Menten model. M ± SEM; n = 9.

Table 5.2 Comparisons of model parameters between temperatures and leaf types using the extra sum of squares F test. Alder leaves (A) and oak leaves (O) were exposed during 18 days in microcosms to 12 ºC or 18 ºC. Model parameters are shown in Table 5.1.

	Comparison		Parameter	F	P
Fungal reproduction	A	12 ºC vs 18 ºC	*Amplitude*	5.15	0.0253
			Center	19.87	<0.0001
Fungal biomass	A	12 ºC vs 18 ºC	V_{max}	22.37	<0.0001
			K_m	4.05	0.0468
	O	12 ºC vs 18 ºC	V_{max}	33.48	<0.0001
			K_m	5.96	0.0163
	12 ºC	A vs O	V_{max}	3.52	0.0636
			K_m	14.25	0.0003
	18 ºC	A vs O	V_{max}	44.40	<0.0001
			K_m	12.83	0.0005
N immobilization	A	12 ºC vs 18 ºC	V_{max}	6.03	0.0197
			K_m	1.68	0.2044
	O	12 ºC vs 18 ºC	V_{max}	5.19	0.0295
			K_m	0.19	0.6626
	12 ºC	A vs O	V_{max}	37.11	<0.0001
			K_m	<0.01	0.9889
	18 ºC	A vs O	V_{max}	44.42	<0.0001
			K_m	0.42	0.5226
Leaf decomposition	A	12 ºC vs 18 ºC	V_{max}	0.47	0.4933
			K_m	21.14	<0.0001
	O	12 ºC vs 18 ºC	V_{max}	33.27	<0.0001
			K_m	1.20	0.2758
	12 ºC	A vs O	V_{max}	572.20	<0.0001
			K_m	14.51	0.0002
	18 ºC	A vs O	V_{max}	765.80	<0.0001
			K_m	1.01	0.3180

5.3.4. Leaf decomposition

Nitrogen concentration in the stream water affected leaf mass loss according to a Michaelis-Menten model (Fig. 5.4, Table 5.1). Maximum leaf mass loss (V_{max}) of alder (66% and 67% at 12ºC and 18ºC, respectively) was higher than that of oak

(29% and 35% at 12ºC and 18ºC, respectively) (P<0.05; Table 5.1, Table 5.2). Maximum leaf decomposition of oak was higher at 18 ºC than at 12 ºC (P<0.05; Table 5.2), but temperature did not change V_{max} of alder leaves (P>0.05). The nitrogen concentration needed to achieve half of maximum leaf mass loss (K_m) of alder was 4-times lower at 18 ºC than at 12 ºC (P<0.05; Table 5.1, Table 5.2), but temperature did not change K_m for oak leaves (P>0.05). At 12 ºC, K_m was lower for oak than for alder leaves (P<0.05; Table 5.2), but at 18 ºC no difference was observed (P>0.05).

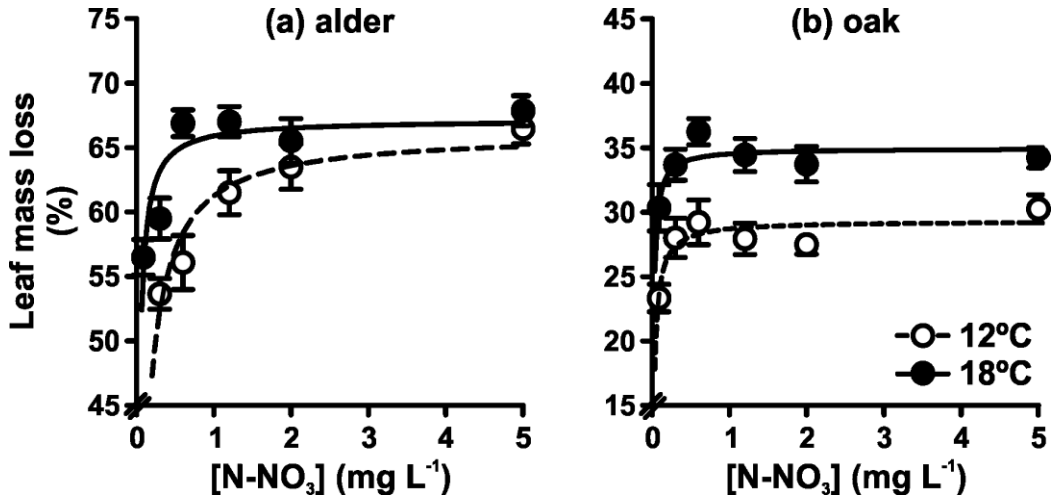

Figure 5.4 Mass loss of alder (a) and oak (b) leaves exposed to increasing N concentrations in the stream water during 18 days in microcosms at 12 ºC and 18 ºC. Data were fitted to a Michaelis-Menten model. M ± SEM; n = 9.

5.4. Discussion

In our study, N enrichment in the stream water led to an increase in N concentration in decomposing leaves, probably as a consequence of N immobilization by the microbial colonizers of leaf litter. Moreover, temperature and leaf type (alder or oak) played an important role in regulating the amount of N immobilized in leaf litter. In our study, a 6 ºC increase in temperature resulted in a 7% increased in maximum N immobilization in alder and oak leaves probably because temperature increased microbial metabolism (Brown *et al.*, 2004; Acuña *et al.*, 2008; Bergfur & Friberg, 2012). In addition, higher N immobilization is anticipated in N-poor detritus or less lignified tissues (Melillo *et al.*, 1984). In our study, microorganisms appeared to immobilize N into a greater extend when thriving on alder leaves which had higher initial N content. Therefore, the increased N immobilization by microbes in alder leaves might have been the result of lower initial lignin concentration in alder leaves (9.1%, Lecerf & Chauvet, 2008) than in oak leaves (19.3%, Lecerf *et al.*, 2007). This

agrees with previous findings reporting that lignin content may have more pronounced effects on microbial activity than those of N content in leaves (Fernandes *et al.*, 2012).

In our study, the Michaelis-Menten kinetics was the one that best explained how N immobilization, fungal biomass and leaf litter decomposition depend on the N concentration in the stream water. This means that these microbially-driven processes increased linearly with the availability of nutrients in the stream water until a certain concentration, above which N is no longer the limiting factor and, consequently, the processes are occurring at its maximum rate. Maximum leaf decomposition (V_{max}) was generally higher in alder leaves, sustaining our hypotheses that leaves with high quality may support higher microbial activity and leaf decomposition (Webster & Benfield, 1986; Sampaio *et al.*, 2001; Lecerf & Chauvet, 2008). Nitrogen concentration in the stream water needed to achieve half of maximum leaf decomposition (K_m) was higher for alder than for oak leaves only at 12 ºC. Conversely, for fungal biomass K_m was lower on alder than on oak leaves, at both temperatures. Because oak has lower initial N content than alder leaves, we expected that higher external N concentration was needed to support microbial activity.

In manipulative experiments with fungal assemblages, a 5-10 °C temperature increase led to faster litter decomposition (Ferreira & Chauvet, 2011a, 2011b; Geraldes *et al.*, 2012). In our study, an increase in 6 ºC led to a 1.2 times increase in maximum mass loss (V_{max}) of oak leaves. However, a 6 ºC increase did not affect V_{max} of alder leaf decomposition, but led to a 4-times decrease of the nutrient concentration needed to achieve half of maximum leaf mass loss (K_m). Maximum fungal reproduction at the higher temperature (18 ºC) was also attained with much less N concentration in the stream water. In addition, K_m values for fungal biomass were 2.4-3 times lower at higher temperature for both leaf types. Nutritional demands of microbes are fulfilled at relatively low levels of inorganic nutrients (Rosemond *et al.*, 2002; Ferreira *et al.*, 2006; Gulis *et al.*, 2006, our study) and in our study increased temperature lowered N concentration needed to attain maximum microbial activity. This suggests that future increases in temperature might result in increased microbially-driven leaf decomposition in oligotrophic streams, especially in those bordered by high quality plant species. This in turn may have implication to benthic invertebrates that feed on decomposing leaves. In our study, temperature enhanced N immobilization in leaves, as well as fungal biomass with lower levels of N in the stream water, and as a consequence leaves may become more palatable to invertebrate shredders and, therefore, we might face

accelerated leaf decomposition in oligotrophic streams under a warming scenario. However, overall impacts on leaf decomposition are difficult to predict because invertebrate detritivores are known to be sensitive to temperature. In 10 first-order Icelandic streams differing in geothermal influence with temperatures ranging between 6 and 23 ºC, macroinvertebrate density increased significantly with temperature, but macroinvertebrate evenness and species overlap decreased (Friberg *et al.*, 2009). Also, an increase in temperature from 10 to 15 ºC led to increased mortality of an aquatic detritivore in microcosms (Ferreira *et al.*, 2010). On the other hand, in nutrient-enriched streams, microbes might not respond to further increases in nutrient because maximum activity was already achieved. Therefore, N concentration in the stream water might increase contributing to eutrophication with negative impacts to sensitive taxa of invertebrate shredders (Pascoal *et al.*, 2001) and further compromising detritus food webs (Woodward *et al.*, 2012).

It is generally expected that nutrients would become toxic above certain concentrations and inhibit biological processes. A recent pan-European experiment conducted in 100 streams encompassing a large nutrient gradient (0.0007-0.926 mg L^{-1} SRP and 0.01-16.9 mg L^{-1} DIN) showed a hump-shaped curve relationship between nutrients in the stream water and leaf-litter decomposition (Woodward *et al.*, 2012). In the current study, leaf decomposition increased asymptotically with increasing nutrient concentration without inhibition even at 5 mg L^{-1} $N\text{-}NO_3$. In eutrophied streams other compounds such as ammonia and nitrite are frequently present (Lecerf *et al.*, 2006), which make it difficult to extrapolate results from microcosms. Nevertheless, in our study the dependence of leaf decomposition on N concentration in the stream water (K_m = 0.02-0.08 and 0.01-0.02 mg L^{-1} $N\text{-}NO_3$ for alder and oak, respectively) was similar to that found in a whole-stream nitrate enrichment study (K_m=0.02 and 0.05 mg L^{-1} $N\text{-}NO_3$ for alder and oak, respectively; Ferreira *et al.*, 2006), suggesting that our microcosms mirrored leaf litter decomposition in streams.

Decomposition may be P-limited at high N:P ratios (Güsewell & Gessner, 2009). In our study, addition of N to stream water created a range of N:P ratios between 30 and 1667. Addition of P led to successive 10-fold decrease of N:P ratio with the lower values ranging from 0.3 to 17. However, leaf decomposition, fungal biomass and reproduction did not seem to be affected by P concentration in stream water. Absence of effects of P enrichment in leaf decomposition in streams was previously observed (Ferreiro *et al.*, 2011) as well as superior effects of N comparing to P concentration (Carpenter & Adams, 1979; Suberkropp & Chauvet, 1995).

Overall, our results suggest that future increases in temperature are likely to potentiate effects of stream water nutrients on microbially-driven leaf decomposition. Moreover, we found that litter quality may interact with the effects of nutrient enrichment and water temperature on plant litter decomposition. Our study further supported that microbial litter decomposition leans on the quality of resources with faster decomposition in high quality leaves. Nutrient enrichment in stream water resulted in an overall increase of fungal activity following a Michaelis-Menten model. The increase in temperature potentiated maximum fungal activity and decreased the nutrient concentration needed to achieve it, especially on high quality leaves. This suggests that in a warming scenario, oligotrophic streams bordered by riparian vegetation delivering high quality leaf litter might be particularly vulnerable to increases in temperature because higher microbial activities might lead to faster organic matter turnover, steeply decreasing the stock of resources for other aquatic organism in streams. Recovering eutrophied streams or protecting oligotrophic ones might help attenuate the effects of future global warming on this important ecosystem process in streams.

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Chapter 6

General discussion and future perspectives

General discussion and future perspectives

Human well-being depends on goods and services provided by ecosystems, namely provisioning services such as food, water, timber, and fibber; regulating services such as the regulation of climate, floods, disease, wastes, and water quality; cultural services such as recreation, aesthetic enjoyment, and spiritual fulfilment; and supporting services such as photosynthesis, and nutrient cycling (Millennium Ecosystem Assessment, 2005). Running waters, which supply essential ecosystem goods and services to humans (e.g. food, water, and waste removal), are heavily impacted by anthropogenic stressors worldwide (Malmqvist & Rundle, 2002) and play a role as key sentinels and integrators of environmental change in the surrounding terrestrial landscape (Williamson *et al.*, 2008).

Headwaters are particularly important to downstream systems as they possess attributes that meet unique habitat requirements for residents and migrants by offering a refuge from temperature and flow extremes; serving as a source of colonists; providing spawning sites and rearing areas; being a rich source of food; and creating migration corridors throughout the landscape (Meyer *et al.*, 2007). As a result, degradation of headwaters and the loss of their connectivity to ecosystems downstream threaten the biological integrity of entire river networks (Meyer *et al.*, 2007).

Plant litter represents a key food source for aquatic biota in low-order forest streams (Vannote *et al.*, 1980), and its inputs depend on the surrounding riparian forest composition which has been altered due to human activities (Graça *et al.*, 2002; Foley *et al.*, 2005; Haines-Young, 2009). In this study, we simulated leaf species loss from a mixture of 3 plant species common in riparian vegetation of the Iberian Peninsula. Leaf mixtures containing the tree plant species were immersed in a stream to allow microbial colonization and then leaf species loss was simulated in microcosms. The experiment was conducted for 6 months to assess the potential impacts of time after leaf diversity loss on microbial diversity and activity, as well as on the feeding behaviour and elemental body composition of a stream invertebrate shredder (Chapter 2 and Chapter 3). Leaf litter diversity loss led to a decrease in microbial diversity (as overall number of OTUs per leaf species treatment) especially of fungi, suggesting that leaf diversity is essential to support more diverse assemblages of microbial decomposers (Chapter 2). In addition, fungal biomass tended to decrease with leaf species loss, especially for lower quality leaf species (oak and eucalyptus; higher C:N ratio) after long time of diversity loss. Leaf decomposition driven by microbes was not affected by leaf species number, but the

presence of eucalyptus (lower quality leaves) lowered the decomposition of alder leaves at the longer time after leaf diversity loss, probably due to the release of inhibitory compounds from eucalyptus leaves, such as essential oils or tannic acid (Canhoto & Graça, 1999). However, decomposition of eucalyptus leaves tended to be faster when mixed with alder leaves (higher quality). It seems likely that microorganisms growing on low quality leaves may benefit from the presence of more leaf species in mixtures or of higher quality leaves, which may contain compounds needed to fulfil their metabolic needs (Gessner *et al.*, 2010). Besides affecting microbial activity, changes in litter diversity may have repercussions to higher trophic levels dependent on plant litter. Indeed, we found that both the number and identity of leaf species affected leaf consumption and FPOM production by invertebrate shredders (Chapter 3). Also, the positive diversity effects on leaf consumption and FPOM production increased with time after leaf diversity loss. This, together with the stronger effects of leaf diversity of microbial activity at longer times (Chapter 2), highlights the importance of time after leaf diversity loss on aquatic biota dependent of plant litter in streams.

Because leaves may have very different chemical composition (Lecerf *et al.*, 2007; Hladyz *et al.*, 2009), changes in litter diversity or quality may result in altered nutrient imbalances between leaf litter and invertebrate shredders. In our study, the C:N imbalance between leaf litter and invertebrates changed with litter diversity. Consequently, leaf consumption by the animals decreased as C:N imbalance increased. In addition, elemental body composition of invertebrates (%C or %N) changed, suggesting that alterations in litter quality may deviate invertebrates from their strict homeostasis. Moreover, FPOM quality correlated positively with leaf quality, which may affect aquatic biota that feed on FPOM. The results from these first studies suggest that: riparian plant diversity is important to microbial diversity and activity (Chapter 2); riparian plant diversity can affect invertebrate shredder activity and quality of resources (FPOM and invertebrate shredders) available to higher trophic levels (Chapter 3). Most importantly, the effects of plant diversity loss on leaf decomposition are likely to become stronger with time (Chapter 2 and Chapter 3). Our results highlight that protection of small headwater streams with diverse riparian vegetation might help to cope with the loss of plant species at downstream sites by draining diverse leaf species that may be transported downstream helping to maintain aquatic biota (Larrañaga et al., 2009) and possibly ecosystem functioning.

Nevertheless, the prediction of potential effects of changes in riparian vegetation on leaf decomposition and associated aquatic biota might be complicated as streams

are simultaneously exposed to several stressors (Malmqvist & Rundle, 2002; Ormerod *et al.*, 2010). For instance, changes in precipitation regimes are expected to occur in the future, as a consequence of ongoing climate change, which may lead to increase in nutrient concentration in streams (Alcamo *et al.*, 2007): higher precipitations can raise surface runoff and erosion leading to increased nutrient concentrations in water bodies (Jeppesen *et al.*, 2009; Jeppesen *et al.*, 2011); and decreased summer flow and increased evapotranspiration may result in less dilution of nutrient inputs and higher nutrient concentrations in water bodies (Murdoch *et al.*, 2000; Whitehead *et al.*, 2009). To evaluate the potential interactive effects of increasing nutrient concentration in streams and plant litter diversity, we carried out a field experiment in which leaves of five species common in the riparian area of the studied sites (alder, oak, chestnut, eucalyptus and plane tree), alone or in selected mixtures, were enclosed in mesh bags and immersed in six streams along a gradient of eutrophication (Chapter 4). Leaf species identity affected leaf mass loss, and biomass of fungi and invertebrates on leaves. The effects of leaf species identity might be explained by differences in litter chemistry, such as nitrogen, phosphorus and lignin concentrations (Lecerf & Chauvet, 2008; Hladyz *et al.*, 2009; Fernandes *et al.*, 2012), which may result in higher or lower quality resources to aquatic biota and, consequently, to higher or lower leaf decompositions. In this study, a positive linear relationship between initial N concentration in leaves and leaf mass loss was found and, interestingly, the slopes of this relationship increased with eutrophication (except for the most eutrophic stream). This suggests that eutrophication may potentiate the effects of leaf identity/quality on leaf decomposition at least until a certain eutrophication level. The lack of relationship between initial leaf quality and decomposition in the most eutrophic stream might be attributed to inhibition of fungal biomass production and the absence of shredders. Leaf mass loss and fungal biomass were higher at the most diverse leaf mixture, but no effects of leaf species number were observed on invertebrate biomass. Leaf-litter decomposition in mixtures was higher than that expected based on the weighted sum of decomposition of individual leaf species, but these diversity effects were lost in the most eutrophic streams. This suggests that eutrophication may attenuate leaf diversity effects on leaf decomposition in streams. Altogether results suggest that nutrient concentration in streams may modulate the effects of leaf diversity on leaf litter decomposition by enhancing the effects of leaf quality and attenuating the effects of leaf species number.

Global warming is an expected consequence of the ongoing climate change (Meehl *et al.*, 2007) which may interact with changes in riparian vegetation and

eutrophication, further disturbing freshwater biota and ecosystem functioning. So in a follow up study, we used naturally occurring aquatic microbial assemblages, under controlled conditions in microcosms, to examine the effects of litter quality (alder and oak, as high and low quality leaf types, respectively), increasing inorganic nutrient concentration and increased stream water temperature on leaf decomposition and activity of the associated microbes (Chapter 5). Nitrogen immobilization in high quality leaves (alder) was higher than in low quality leaves (oak) and increased with N concentration and temperature in the stream water for both leaf types. Overall, microbial activity increased asymptotically (Michaelis-Menten kinetics) with N concentration in the stream water. Although nutrients may become toxic above certain concentrations and inhibit ecological processes such as leaf decomposition in streams (Woodward *et al.*, 2012), we found no sign of inhibition of microbial activity with increasing eutrophication, with the exception for fungal reproduction on alder leaves, which was inhibited by high N concentration (>3 mg L^{-1} $N\text{-}NO_3$ at ambient temperature) in the stream water. We found that leaf decomposition depended on N concentration in the stream water in a similar way (Michaelis-Menten kinetic) as in a whole-stream nitrate enrichment study (Ferreira *et al.*, 2006), suggesting that our microcosm approach mimic well leaf litter decomposition in streams. The inhibition of leaf decomposition reported in very eutrophic streams may result from the presence of other compounds such as ammonia and nitrite that generally are toxic and co-occur in eutrophic systems (Lecerf *et al.*, 2006).

As expected, increased temperature led to an increase in maximum fungal activity, given that organism metabolism tends to increase with increasing temperature (Brown *et al.*, 2004; Acuña *et al.*, 2008). Interestingly, increased temperature led to a decrease in the N concentration needed to achieve maximum fungal activity, especially on alder leaves. This suggests that, under the predicted warming scenario, maximum fungal activity may be attained in streams with lower nutrient levels, especially those receiving high quality leaf litter, and this may result in faster leaf decomposition.

Overall our results show that changes in leaf diversity affected microbial activity and diversity, invertebrate feeding rate and the production of FPOM, and effects tended to become stronger at longer times (Chapter 2 and 3; Fig. 6.1). Leaf diversity loss may promote decreased fungal diversity (Chapter 2), which may result in direct effects on leaf decomposition (Duarte *et al.*, 2006) and on indirect effects through invertebrate preferential feeding on certain fungal species on leaves (Jabiol & Chauvet, 2012). It would be important to further assess which microbial species are associated with specific leaf species and which are the potential effects of their loss

on leaf decomposition and on higher trophic levels. In addition, FPOM quality and elemental composition of invertebrate shredders changed with litter quality (Chapter 3). It would be important to further study whether changes in basal resources quality (leaf litter) can affect higher trophic levels of the detritus dependent food web in streams besides invertebrate shredders.

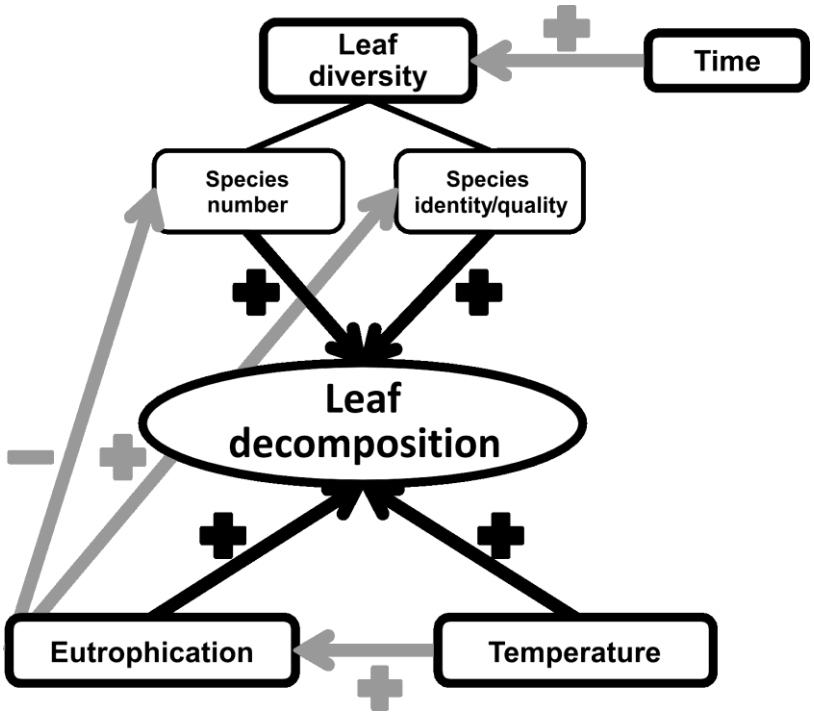

Figure 6.1 Conceptual diagram depicting potential interactive effects of the main factors affecting leaf decomposition in streams tested in the present study: leaf diversity (as number and identity of leaf species), time after leaf diversity change, eutrophication and temperature. Black and grey arrows indicate direct and indirect effects, respectively; the signs plus (+) or minus (-) indicate stimulation or inhibition effect, respectively.

Stream eutrophication modulated leaf diversity effects on leaf decomposition by decreasing the effects of leaf species number and enhancing the effects of leaf quality (Chapter 4; Fig. 6.1). Furthermore, future increases in temperature are likely to potentiate the effects of stream water nutrients on microbially-driven leaf decomposition, especially in the most oligotrophic streams (Fig. 6.1). Altogether, the interactive effects of plant litter diversity, increased eutrophication and temperature in streams may lead to significant changes in key ecological processes such as leaf litter decomposition and associated biota. In order to minimize the potential impacts of these effects, future actions should focus on: i) protecting non-impacted headwater streams from anthropogenic activities resulting in eutrophication and maintaining the natural riparian vegetation; ii) restore riparian corridors and further control and reduce eutrophication levels at impacted sites. European Union is

committed in reducing the release of nutrients into water bodies (Nitrates Directive, 1991/696/EC) and aims at achieving a good ecological condition of groundwater and surface waters until 2015 (Water Framework Directive, 2000/60/EC). This is a challenging and difficult task and current assessments estimated that at least 40% of the EU's surface water bodies are at risk of not meeting the 2015 objectives (WISE, 2008). Also, forecasted climate change effects on the diversity and distribution of higher plants in Europe predicted that 32% of plant species might be lost by 2050 (Bakkenes *et al.*, 2002). This together with a careless land use might lead to major changes in plant community composition and thereby to alterations in the quality of plant litter available to aquatic biota. Given that we live in an increasingly connected world and considering the global nature of many environmental issues, new collaborative approaches, controlling both spatial and temporal scales and encompassing large geographic ranges, involving standardized protocols, are needed if the scientific community wants to advance their understanding of general principles in ecology and environmental science (*Peters et al.*, 2008; Fraser *et al.*, 2013).

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